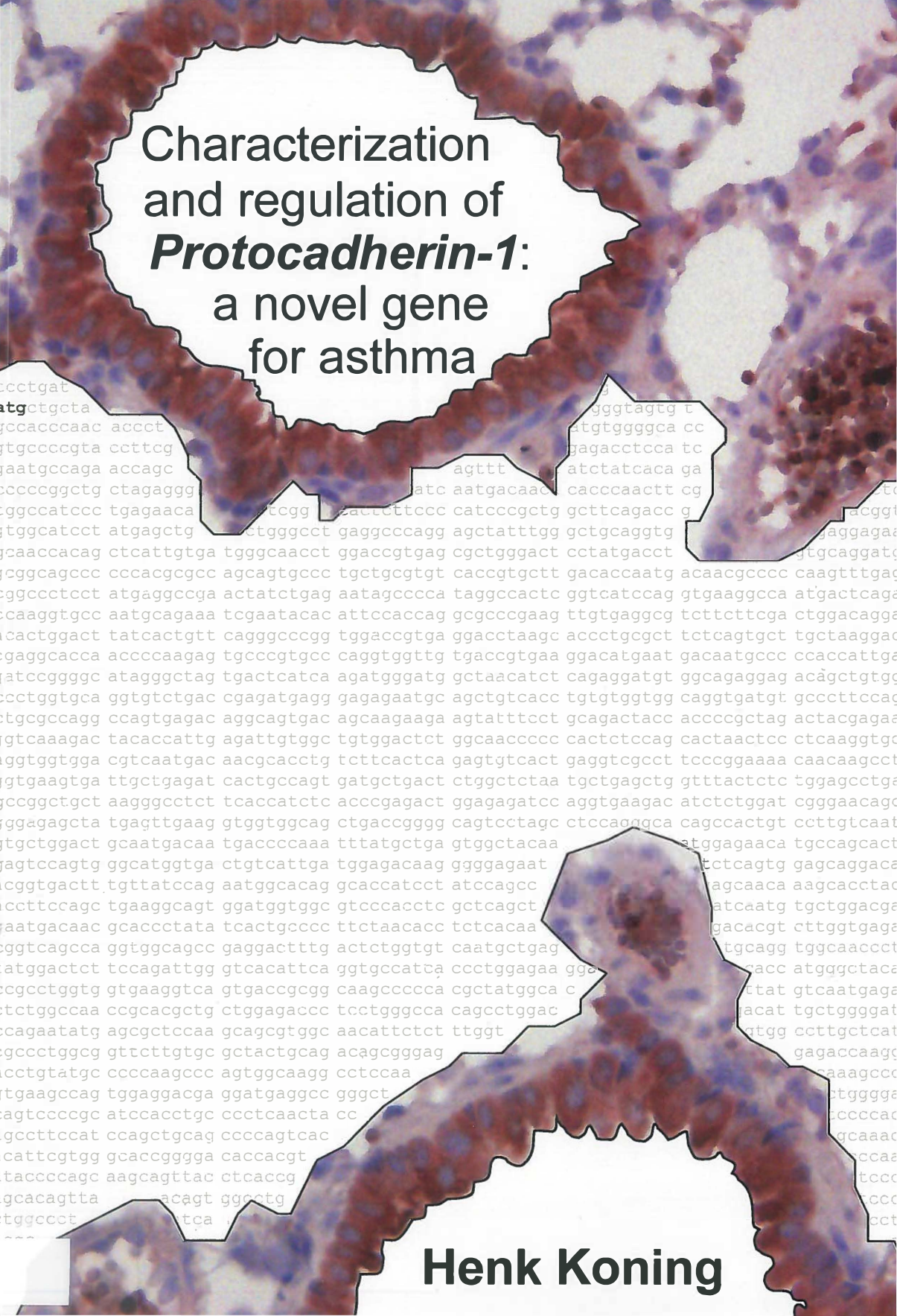
Characterization and regulation of Protocadherin-1

Koning, Henk


Characterization and regulation of *Protocadherin-1*: a novel gene for asthma
Henk Koning

Characterization and regulation of *Protocadherin-1*: a novel gene for asthma

Stellingen

Behorende bij het proefschrift

Characterization and regulation of *Protocadherin-1*: a novel gene for asthma

1. De functie van Protocadherine-1 kan beter onderzocht worden in cellen die gekweekt worden op Air Liquid Interface dan in cellen die gekweekt zijn in een monolayer (dit proefschrift).

2. Protocadherine-1 is geen klassiek adhesiemolecuul, maar kan het best worden gezien als een adhesiemolecuul met signaleringsmogelijkheden (dit proefschrift).

3. De specificiteit van een antilichaam voor immunohistochemie of immunofluorescente kleuringen, zonder duidelijke positieve controle, kan slechts met zekerheid worden vastgesteld door middel van knock-out muizen of knockdown experimenten (dit proefschrift).

4. Het integreren van kennis over verschillende astmagenen, zoals *PCDH1* en *SMAD3*, kan leiden tot nieuwe inzichten in de pathogenese van astma (dit proefschrift).

5. Men moet niet de biologische interpretatie van een gen-gen interactie verwarren met de interpretatie van een biologische interactie tussen de eiwit-producten van twee genen.

6. Mensen zijn objecten die gebrusht dienen te worden

7. Een wetenschapper is net zo afhankelijk van zijn toolbox als een timmerman.

8. Op de racefiets heeft ook de voorste van een peloton voordeel van het fietsen in een groep (Bert Blocken, TU Eindhoven).

9. Er wordt minder roofvis gevangen wanneer hetzelfde kunstaas meermaals gepresenteerd wordt in het hetzelfde viswater.

Henk Koning 21 november 2012

Paranimfen:

Uilke Brouwer
Frans Koopmans

This research was funded by:
A GABRIEL studentship (a multidisciplinary study to identify the genetic and environmental causes of asthma in the European Community, a European Commission FP6 grant), and the UMCG Groningen

Printing of this thesis was financially supported by:

rijksuniversiteit groningen
GUIDE Research Institute for Drug Exploration
Takeda Nederland BV
TEVA Nederland
NOVARTIS
Boehringer Ingelheim
greiner bio-one
Products: PCR-plates 96-wells
Stichting Astma Bestrijding

Cover design and layout: Henk Koning
Printing: Off Page, Amsterdam

ISBN: 978-90-367-5865-9

cmb.

rijksuniversiteit groningen

Characterization and regulation of *Protocadherin-1*: a novel gene for asthma

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
woensdag 21 november 2012
om 12.45 uur

door

Henk Koning

geboren op 17 mei 1983
te Leeuwarden

Promotores:	Prof. dr. G.H. Koppelman Prof. dr. A.J.M. van Oosterhout Prof. dr. D.S. Postma
Copromotor:	Dr. M.C. Nawijn
Beoordelingscommissie:	Prof. dr. J. Holloway Prof. dr. P. Hiemstra Prof. dr. F. van Roy

Ter herinnering aan

Mijn broer Anne-Jan Koning †27-3-2009
en vader Jan Koning †7-02-2012

Die te vroeg van ons zijn heengegaan

Table of contents

Chapter 1

General Introduction and Aims of this Thesis

OVERVIEW

In this thesis we aim to investigate the role of Protocadherin-1 in asthma pathogenesis. Therefore, this introduction first describes the general and clinical background of asthma and bronchial hyperresponsiveness (BHR) and the basic immunological background of asthma. Next the normal structure of airway wall is being discussed, followed by pathological features of asthma. Then the contribution of the inflammatory and structural changes of the airways to BHR will be discussed. Despite our current knowledge on the inflammatory and structural background of asthma, the exact mechanisms leading to BHR and asthma are unknown. Genetic studies are conducted in order to identify genes, the identity of which might inform on new mechanisms in asthma. Therefore in the last sections, the current genetic knowledge of BHR and asthma is described, and the family of protocadherins is introduced. Finally the rationale and the research questions of this thesis are presented.

Asthma and Bronchial hyperresponsiveness

Asthma is a common chronic disease of the airways affecting up to 300 million people world-wide, of different ages and ethnic backgrounds (1). Most of asthma has its onset in childhood (2). Asthma is characterized clinically by variable airway obstruction with a reversible component, and BHR triggered by smoke, cold air or allergens like house-dust mite (3). Asthma patients experience episodes of cough, wheezing and shortness of breath. Furthermore, airflow limitation may result in severe shortness of breath or induce exacerbations (4). Asthma is a phenotypically heterogeneous disease, which is preceded by varying patterns of wheezing in childhood (5). In addition, asthma has a marked variation in age at onset, severity and progression of the disease; some children with mild asthma can remit from the disease, while most of the severe asthmatic children still suffer from asthma in adulthood (6). Moreover, a proportion of asthmatics develops asthma at adult age, e.g. after menopause in females (7).

To date, asthma is controlled by anti-inflammatory drugs such as glucocorticoids and leukotriene antagonists, whereas bronchoconstriction is effectively reduced with bronchodilators such as short- or long-acting β2-agonists and anticholinergics. Combination therapies of glucocorticoids and long acting β2-agonists may increase asthma control by

improving lung function and reducing exacerbations (8). For control of severe asthma, add-on therapy with anti-IgE can be considered (8). However, while these treatment options effectively suppress the symptoms of the disease, they fail to cure asthma. Furthermore, deterioration of clinical control may occur if these medications are discontinued (8). As the exact causes of asthma are unknown, a treatment option that can cure or prevent asthma has not yet been developed.

One of the main characteristics of asthma is bronchial hyperresponsiveness (BHR). BHR is defined as a heightened responsiveness of the airways to a range of different non-allergic stimuli, and is generally quantified by a provocative agent which induces a 20% fall (PC_{20}) in the forced expiratory volume in one second (FEV_1). Given the way BHR is expressed, BHR is associated with pre-challenge FEV_1. In the general population BHR has a population prevalence of up to 35%. BHR is present both in subjects with (symptomatic BHR) and without respiratory symptoms (asymptomatic BHR) (9). Asymptomatic BHR is a predictor for development of respiratory diseases, such as asthma (10).

BHR can be detected by administration of histamine or methacholine that act by stimulation of H_1- or muscarinic receptors of airway smooth muscle cells. Furthermore, indirect methods of determining BHR are available, such as exercise, exposure to cold air, hypertonic saline, mannitol, or adenosine-mono-phosphate (AMP) (11). These indirect factors generally target immune cells like mast cells, which in turn release mediators like histamine, leukotrienes or prostaglandins stimulating airway smooth muscle contraction. Therefore, the indirect method of detecting BHR more closely reflects the inflammatory status of the airways, which is especially relevant when testing the effect of immunomodulating drugs on BHR (12, 13). In order to get a better understanding of the immunological background of BHR and asthma, a brief overview of the mechanisms in asthma is provided in the next section.

Mechanisms in asthma: the interface of immunological and structural cells of the airway

Immunological mechanisms in allergic asthma

Most asthma is characterized by an underlying allergic response to inhaled innocuous substances called allergens. When allergens are inhaled and enter the airways, they are taken

up by dendritic cells (DCs). After DCs have taken up an antigen, they can migrate to the draining lymph nodes where naive T-cells reside, and present the antigen in the context of Major Histocompability Class (MHC)-II molecules. Migration of DCs towards the draining lymph nodes and antigen presentation by the DCs is regulated by the local tissue microenvironment, consisting of stromal cells like fibroblasts, endothelial and epithelial cells (14). House-dust mite allergens for example, activate pattern recognition receptors on airway epithelial cells. Epithelial cells in turn produce cytokines like TSLP, IL-33 and IL-25 that activate DCs, indicating that the airway epithelium plays a regulatory role in the induction of a response to inhaled antigens (15). Antigen-presenting DCs can either activate T-cells so that they differentiate towards effector phenotypes such as T_H1, T_H2 and T_H17 cells, induce a tolerogenic or a regulatory T-cell phenotype. The outcome of the DC-T cell interaction in terms of T-cell phenotypes is a function of the DC activation state at the time of the cognate interaction and the cytokines present in the microenvironment (16).

Once activated, T_H1-cells produce interleukin (IL)-2, Tumor Necrosis Factor alpha (TNF-α), and interferon gamma (IFN-γ), and are critical in the development of cell-mediated immunity, in which macrophages, natural killer cells (NK), and antigen-specific cytotoxic T-lymphocytes work together to clear intracellular pathogens (16).

T_H2-cells produce a range of different interleukins such as IL-4, IL-5, IL-9, and IL-13. IL-4, together with IL-13, induces B-cells to produce IgE-antibodies against the selected antigen or allergen. Furthermore IL-4 induces maturation of mast cells, which start expressing specific receptors for these IgE molecules (FcεRI-receptors). IL-5 specifically attracts eosinophils (17). IL-9 shares many features with IL-4, and assists in growth of T-lymphocytes and maturation of mast cells (18). In a sensitized individual, that has allergen-specific IgE antibodies, renewed exposure to an allergen will induce an allergic response. IgE-loaded mast cells become activated and degranulate, resulting in the immediate release of several pro-inflammatory mediators like leukotrienes, and histamine, which trigger airways constriction. This early phase of the allergic reaction is characterized by increased vascular permeability and mucosal oedema (19). After 3-6 hours, this facilitates the entry of inflammatory cells, like T_H2-cell and eosinophils into the lung parenchyma and airway epithelium. The activation of these cells and the subsequent release of pro-inflammatory cytokines, leukotrienes and damaging proteins like major basic protein, eosinophil cationic protein, and eosinophil peroxidase causes a second period of airway narrowing, the so-called late phase response to allergens (19, 20). T_H17-cells produce cytokines of the IL-17 family like IL-17A and IL17-F. These cytokines act on a range of cell-types such as epithelial cells, endothelial cells and

fibroblasts, and induce the production of cytokines like IL-8 that subsequently attract neutrophils, amongst others assisting in the defense reaction against extracellular pathogens and fungi (21).

Regulatory T-cells (Tregs) suppress the activity of effector T-cells and thereby maintain tolerance towards self-antigens and inhaled allergens (22). The induction of effector versus tolerogenic T-cell phenotypes is critically dependent on the DC phenotype (immature and mature DCs) and the microenvironment at the time of antigen presentation, and determines the induction of tolerance versus immunity towards the inhaled allergen (23). In the absence of signals inducing DC maturation and expression of costimulatory markers, presentation of airway-derived antigens in the draining lymph nodes will result in regulatory T-cell (Treg) induction and tolerance. Tregs maintain tolerance in an antigen-specific fashion. Tregs can impair the capacity of DCs to prime naive T-cells towards effector phenotypes, thereby promoting the development of tolerogenic DCs. Furthermore, Tregs can directly inhibit the activity of effector T-cells (16). A dysbalance in Treg-activity may lead to a decreased suppressive activity and thereby to an increased inflammatory response (24).

Asthma has originally been postulated to be a disease characterized by a T_H2-type of inflammatory response (25). But as increased levels of IL17-A are detected in lungs of asthmatic patients compared to healthy controls, T_H17 effector cells may also play a role, and may explain the presence of low numbers of neutrophils in asthma (26). Furthermore, the presence of lower numbers of Treg cells in asthmatic patients compared to controls may lead to a decreased suppression of inflammatory cells and effector T-cells (22), thereby inducing an aggravated inflammatory response. Together with a continuing exposure to allergens, this may lead to a chronic inflammatory response in the airways. The chronic inflammatory response to seemingly harmless agents is in turn thought to contribute to the remodelling of the asthmatic airways. To understand the process of airway remodelling in the asthmatic airways, the resident cells of the airway wall are described in the next section.

Resident cells of the airway wall

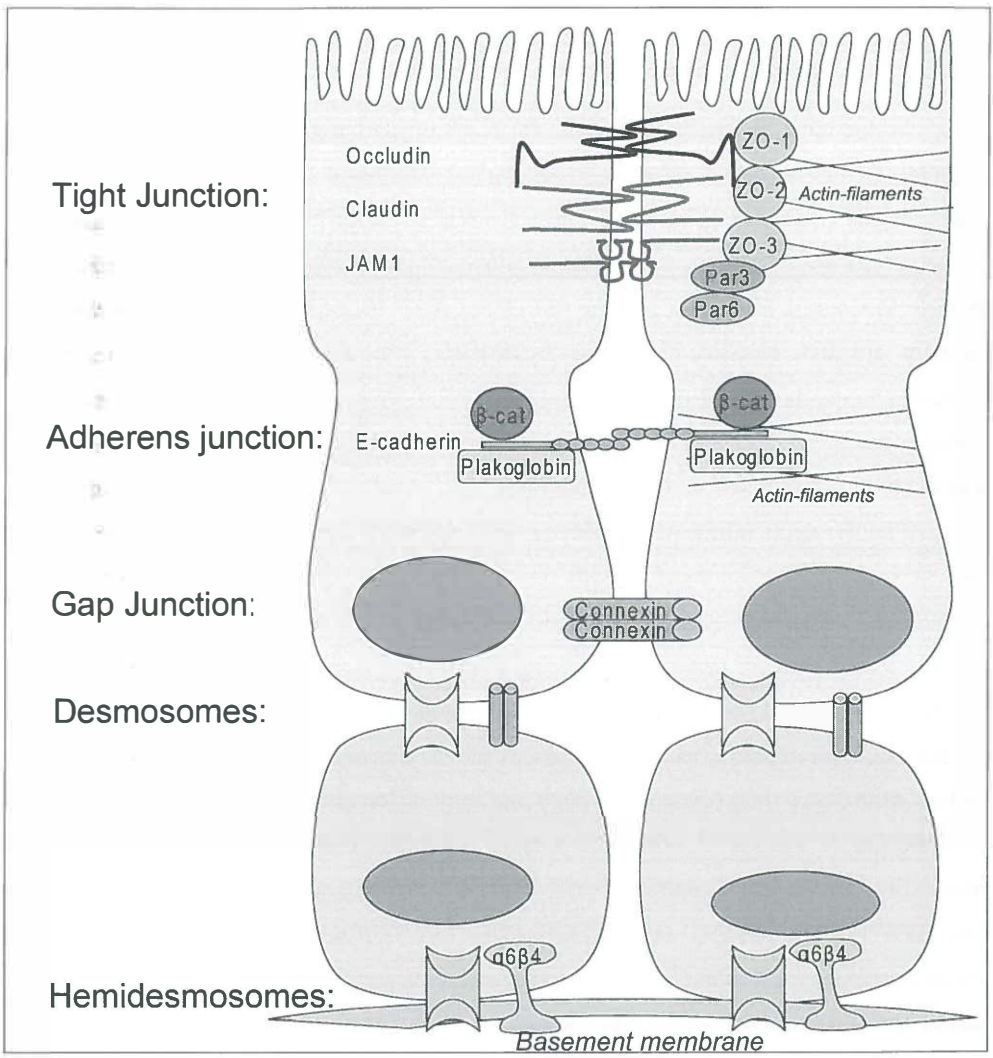
Under physiological conditions, the airway wall is lined by a pseudo-stratified epithelial cell boundary, protected by a mucous layer. This thin layer of viscous fluid is produced by secretory epithelial glands. Epithelial cells are located on top of the reticular basement membrane, a supportive structure consisting of collagen, laminin and proteoglycan molecules (27). The area below the basement membrane is called the lamina propria or submucosa, where fibroblasts are present. Fibroblasts are key players in the maintenance and

turnover of extracellular matrix components, the main constituents of the connective tissue of the submucosal layer (28). The submucosa is positioned in between the basement membrane and smooth muscle cell layer. Smooth muscle cells contribute to the contractility, and by producing extracellular matrix components, to the stability of the airway wall (29). This thesis focuses on two main cell-types in the airway wall important for asthma and BHR; the airway epithelium and airway smooth muscle cells.

The airway epithelium is the first line of defence against invading pathogens, inhaled allergens or irritant agents, such as house-dust mite or smoke, respectively. It forms a physical barrier by maintaining a size- and ion selective impermeable connections between neighbouring epithelial cells, and by the production of a mucous layer. Epithelial cells connect to each other by tight-and adherens junctions, formed by adhesion molecules like Occludin, Claudins, JAM1, Zona-Occludins (ZO) 1, 2 and 3 (all of which localize to the tight-junctions), and E-cadherin, β-catenin and plakoglobin (all of which localize to the adherens junctions) (30). Communication between cells takes place by gap junctions, which are build-up by connexins, and facilitate the transport of molecules between cells (31) (Figure 1). This thesis discusses the epithelial cells of the large airways, as these are studied most extensively, and are subject of our studies. In the airway epithelium of the large airways several cell types have been identified, which can be categorized into four main categories: basal, undifferentiated columnar, secretory and ciliated cells (32). Basal cells are strongly attached to the basement membrane by hemidesmosomes and integrins and thereby provide support for columnar or differentiated epithelial cells. Attachment of basal cells to the columnar or differentiated cells is facilitated by desmosomes (33). These structures consist of several integral membrane proteins like desmocollin and desmoglein, and adaptor molecules plakophilin and plakoglobin, which connect to intermediate filaments and thereby maintain the integrity of the airway epithelium (Figure 1; (34)). Furthermore, basal cells are considered as the progenitor cell-type in the lung (35, 36). Secretory cells consist of mucous or goblet cells, and Clara cells, mainly secreting mucin granules, antimicrobial proteins and surfactant molecules (36, 37). Besides these cell types, a rare secretory cell exists in the large airway; the neuroendocrine-cell (NEC). NECs secrete surfactants, perform chemoreception (hypoxia sensing) and are involved in the regulation of lung maturation and growth (38, 39). Ciliated cells are the most prominent cell-type in the large airways and their coordinated ciliary beating results in mucociliary clearance.

Figure 1: Structure of the airway epithelium

Basal epithelial cells are connected by integrins (α6β4) and hemidesmosomes to the basement membrane. Communication with columnar, ciliated or other differentiated cells takes place using gap-junctions consisting of connexins. Basal cells do not communicate with each other but provide strong support for differentiated cells. Basal cells are strongly connected to differentiated cells by desmosomes. Differentiated (ciliated) cells communicate by gap-junctions, and are strongly attached to each other by adherens junctions (E-cadherin, β-catenin and plakoglobin) and tight-junctions (Occludins, Claudins, JAM1, ZO-1, 2, 3 and Par3,6). Adaptor molecules like β-catenin (β-cat), plakoglobin, ZO-1, 2, 3 and Par3, 6 provide anchorage to the actin cytoskeleton. Adapted from Nawijn et al., Trends in Immunology, 2011 (30).



Besides the physical barrier function, the airway epithelium has a sensory innate immune function. Airway epithelial cells can sense the presence of bacteria, viruses, fungi or components of these organisms, also known as pathogen-associated molecular patterns (PAMPs), via pattern recognition receptors, of which the family of Toll-like receptors (TLRs) are the most well-known receptors (40). Most of the TLRs can sense extracellular PAMPs, but some receptors can sense intracellular PAMPs as well by connection to endosomes (TLR-3, 7/8, 9) (41). TLR-receptor activation results in the expression of pro-inflammatory cytokines, like Interleukin-6 (IL-6), IL-8, TNFα, Interferons (42), TSLP (43), and chemokines like CCL5/RANTES (44). These pro-inflammatory cytokines can recruit inflammatory cells like macrophages and dendritic cells (DCs) for phagocytosis of bacteria and other pathogens, and T-cells for initiation of a T_H1 or T_H2-response.

Furthermore the epithelium has an immune alarming function. Besides PAMPs, originating from infectious micro-organisms, pathogenic insults on the epithelium, injury or trauma of the airway epithelium can induce the release factors alarming the immune-system. These factors originate from the epithelial cells itself and are collectively called alarmins or damage associated molecular patterns (DAMPs) (45). Examples of endogenous epithelial alarmins are high mobility group box 1 (HMGB1) (46) and IL-33 (47), which are both expressed in the nucleus of the airway epithelial cells. Release of these DAMPs can alert the immune system to tissue damage, and warn for possible infections, such that the appropriate immunological responses can be initiated (48).

Finally, upon injury of the airway wall, epithelial cells increase the production of growth factors, like fibroblast growth factor (FGF)-2, insulin-like growth factor (IGF)-l, platelet-derived growth factor (PDGF), endothelin (ET)-1 and TGF-β2, and the expression of apically located growth factor receptors like Epidermal growth factor receptor (EGFR) (49). By secreting these factors and upregulating growth factor receptor expression levels, the epithelium communicates with fibroblasts in the submucosa. Under the influence of these factors, fibroblasts then become activated and may differentiate into myofibroblasts, which may migrate to the site of injury and secrete extracellular matrix components to assist in repair (50). Together with epithelial cells they constitute the epithelial mesenchymal trophic unit (EMTU). This EMTU and its EGF, FGF, and TGF receptor signalling between the epithelium and mesenchyme has been shown to be essential for embryonic lung development, and is reactivated upon airway epithelial injury (51).

The airway smooth muscle (ASM) cells contribute to BHR and asthma due to their role in airways constriction. Under physiological conditions smooth muscle cell functions include

migration, differentiation and contraction. During lung development smooth muscle cells migrate and differentiate to add to the formation of the airway wall (52). Smooth muscle cells contain the contractile proteins actin, myosin, and tropomyosin. Smooth muscle cell contraction results from the interaction of actin filaments with myosin and tropomyosin filaments, in response to a change in calcium concentrations (53). In full-grown lungs of healthy persons, ASM is believed to perform peristalsis, a coordinated symmetrical contraction and relaxation of muscles, in order to assist in exhalation or mucous removal. Furthermore ASM is believed to promote blood flow and ventilation, to protect the peripheral lung and airway structure from toxic substances, and to stabilize the airways (54). Perhaps a novel role for ASMs lays in its communication with other cell-types of the lung; such as airway epithelial cells, where ASM can influence the repair process of the EMTU in mice by secreting the fibroblast growth factor 10 (55).

Remodelling of the airways in asthma

Pathologically, asthma is characterized by airway inflammation and airway remodelling. The main inflammatory cells found in the airways of asthmatics are eosinophils and neutrophils, and to a lesser extent the mast cell (56), as was described before in the chapter regarding the basic inflammatory mechanisms in asthma. Remodelling refers to "*modifications to the normal composition and structural organization of a certain tissue, which occurs in response to various mechanical or physiological forms of stress*" (57). In asthma, airways remodelling is characterized by epithelial changes, subepithelial extracellular matrix deposition, smooth muscle hyperplasia and hypertrophy, increased numbers of activated fibroblasts, and vascular changes (57). The increase in extracellular matrix products, like collagen, fibronectin and tenascin produced by fibroblasts, can lead to thickening of the reticular basement membrane (RBM) (58-60). An increase in blood vessels in the asthmatic airways and increased microvascular permeability are also highly relevant mechanisms in the pathogenesis of asthma that are increasingly attracting attention (61). Changes to the airway epithelium and smooth muscle cell layer in asthma are described in detail below.

What is wrong with the airway epithelium in asthma? In the airway epithelium of asthma patients, several abnormalities have been observed when compared to healthy subjects that relate to structural differences, changes in epithelial cell-type, differences in cytokine and growth factor secretion, increased permeability and an aberrant responses to injury. As evidenced by histopathological studies, epithelial cells are shed from the basement membrane or disconnected from the basal epithelial cell-layer (62), and can be detected in sputum of

asthmatic subjects as creola bodies (63). This epithelial desquamation has been under debate, as it might in part be the result of a sampling artefact (64), but still may reflect a weaker attachment of epithelial cells to the basal cells or basement membrane in asthmatic subjects (65). Besides epithelial denudation, lower expression levels of adhesion molecules have been described in asthmatic epithelium (66-68), altogether suggesting that the integrity of the epithelium is compromised. Interestingly, bronchial epithelial cultures of asthmatic subjects show a decreased barrier function in measurements of transepithelial electrical resistance (TEER) compared to healthy controls (67), and this increased permeability correlates with lower tight-junction molecule expression (69). As such, the observed decrease in adhesion molecule expression can be directly correlated to a decrease in epithelial barrier function of the asthmatic epithelium.

Furthermore, ciliated airway epithelial cells isolated from asthma patients exhibit a reduced ciliary beat frequency, especially in moderate and severe asthmatics (70) when compared to healthy controls. In the asthmatic epithelium there is an increase in goblet cell numbers, mucin mRNA expression levels, and stored mucin (71). Together with the reduced clearance by ciliated cells this could lead to excess of resident mucous in the airways, resulting in mucous plugs and more severe airway obstruction. These mucous plugs or casts can even result in fatal asthma attacks (72).

In addition to these structural changes, asthmatic epithelial cells intrinsically produce increased amounts of cytokines like IL-6 (specifically by epithelial cells of children) (73), IL-8 (68) or TSLP (adults) (74), thereby attracting higher numbers of inflammatory cells like neutrophils, and maintaining a pro-inflammatory response.

Asthmatic bronchial epithelial cells also produce larger amounts of epidermal growth factor (EGF) (73), and TGFβ (75), and upregulate expression levels of its receptors like EGFR (76). It is thought that the EMTU, important in lung development and repair, is abnormally activated in asthma, due to the increase in the production of these growth factors and their receptors (77, 78). This might lead to a continuous repair of the airway epithelium. Epithelial repair upon injury consist of several processes, starting with migration of cells to the site of injury, division of the migrated cells and finally differentiation towards either ciliated or secretory functional cell types (79). The potential defects in repair and differentiation of the asthmatic airway epithelium may, in addition to the structural changes and increased inflammatory status of the epithelium, lead to a severely remodelled epithelium (80), as well as an enhanced susceptibility to environmental challenges such as viruses, allergens and cigarette smoke.

What is wrong with the airway smooth muscle in asthma? In asthma, ASM has undergone changes that may contribute to airway remodelling and BHR. Several studies have identified an increase in airway smooth muscle cell number (hyperplasia) and an increase in cell size (hypertrophy) in the asthmatic airway (81-83). Increases in ASM cell size and cell number result in an overall increased ASM mass. ASM of asthmatic individuals produce increased amounts of extracellular matrix components like collagen I and perlecan, but decreased amounts of laminin-α1 and collagen IV (84). In addition, increased amounts of ECM have been observed around ASM-bundles in the asthmatic airway (85). This increase in ECM, either coming from the ASM itself, or of other origin like epithelial cells and fibroblasts, can induce increased proliferation of airway smooth muscle cells (86). ASM cell proliferation can also be induced by several growth factors and inflammatory mediators from a range of cell types, like histamine of mast cells (86, 87). Furthermore it is hypothesized that ASM cells have an increased migratory phenotype, as *in vitro* an increase in migration is observed after stimulation with cyclic AMP mobilizing agents (88). In addition to the increase of airway smooth muscle mass in asthmatic subjects, the ASM also displays an enhanced sensitivity towards contraction (89). Significantly increased shortening of smooth muscle was detected in ASM of asthmatic subjects when compared to healthy subjects (90, 91). Overall, the increase in proliferation and migration of ASMs are thought to be a major contributor to the increase in ASM mass observed in asthmatic patients, and these may in combination with the increased ASM shortening, be very relevant to bronchoconstriction of the asthmatic airway.

Contribution of inflammatory and structural components to BHR

BHR is hypothesized to consist of a persistent and a variable component, based on results from studies with histamine, methacholine, and indirect measurements of BHR (13). The persistent component is generally thought to be caused by structural changes of the airways collectively referred to as airways remodelling (92), although some of these changes, like thickening of the reticular basement membrane, can be partially reversible (93). Additionally, the variable component of BHR is attributed to airways inflammation, and therefore considered to be reversible upon anti-inflammatory treatment (94). Controversy exists on whether the inflammatory changes are a larger contributor to BHR than the structural changes.

First the contribution of the inflammatory components to BHR is discussed. An allergic reaction to allergen exposure is characterized by an early and late phase response as described previously. When BHR was determined before and at 3h, 24h and 48h after the allergen exposure, it became evident that the development of the late asthmatic response was accompanied by an increase in BHR (four-fold decrease in PC_{20} to histamine) at 3 hours after allergen challenge (95, 96). The increase in BHR both preceded and highly correlated with the magnitude of the late-phase asthmatic response (95, 96). In addition, the BHR remained increased up to 48h after the allergen exposure, emphasizing the close correlation between BHR and the late asthmatic response (95).

Second, when the inflammatory component in asthma is treated for longer periods (i.e. 6-8 weeks, up to 2 years) by anti-inflammatory corticosteroid therapy, an improvement in bronchial hyperreactivity was detected in several studies (93, 97-101). In addition lower numbers of inflammatory cells like eosinophils and mast cells, and a decrease in RBM thickness were detected (93, 98). Mast cells can release several factors like biogenic amines (histamine, serotonin), cytokines, proteoglycans, and various proteases (chymase, tryptase, carboxypeptidase), thereby dilating vessels, and initiating innate immune functions and allergic reactions. (102). Eosinophils can secrete factors like major basic protein (MBP), eosinophilic cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil-derived peroxidise (EPO) (103). These factors may contribute to the contractility and proliferation of ASM, and thereby to BHR (102, 104), or might damage the integrity of the airway epithelium. Therefore, their reduction after anti-inflammatory therapy is important for asthma treatment. Clinical studies specifically targeted at reducing eosinophil numbers (anti-IL-5 therapy), decreased the number of exacerbations of asthma patients, but no improvement in BHR was observed (105, 106). The results from allergen challenges and anti-inflammatory therapies with corticosteroids suggest that the inflammatory component can make a large contribution to BHR, although further studies are needed into the exact role of the inflammatory cell types separately, like eosinophils, in asthma pathogenesis.

Next the contribution of the structural components to BHR is being discussed. One important structural component is the airway smooth muscle, which may by its increase in mass and contractility in asthmatic subjects contribute to BHR. The exact mechanism by which the increase in ASM mass contributes to BHR is unclear. The contribution of smooth muscle to BHR has been estimated and these studies suggest that the increase in smooth muscle mass could be the most important factor for the increase in BHR in asthma (107), but

in vivo studies provide conflicting results. In a mouse model of asthma it was shown that the smooth muscle layer thickness explains a considerable part of methacholine hyperresponsiveness (108). In contrast, Siddiqui and co-workers show that ASM mass does not correlate with BHR to methacholine, but that instead the numbers of ASM resident mast cells per mm^2 ASM correlate with BHR (109). In addition, James *et al* show that degranulation of mast cells correlates with asthma severity (110). In human, Tsurikisawa and co-workers show a positive correlation of ASM thickness with BHR to histamine, but not with BHR to acetylcholine (111). As histamine and acetylcholine target different receptors on the ASMs to induce bronchoconstriction (respectively the H_1-Receptors and the muscarinic-receptors (111)), different pathways leading to BHR may be the underlying cause for these differences. As mast cells can contribute to both cell proliferation, by histamine and tryptase release, and contraction of ASM (104), further studies are needed to identify the exact contribution of ASM and mast cells to BHR.

Another structural component of the airway is the airway epithelium. As previously discussed, the airway epithelium has potential defects in its repair, differentiation and barrier function, but it can also contribute to BHR. In airway wall biopsy studies it is shown that eosinophilic infiltration in the airway epithelium correlates with an increase in opening of epithelial tight-junctions and intercellular spaces, suggesting that eosinophils contribute to epithelial damage (112), which in turn contributes to BHR (113). As the primary function of the airway epithelium is to act as a cellular barrier between the air and lower located cell types, such as smooth muscle cells, a damaged airway epithelium with a weaker barrier function may thereby increase the sensitivity of the airways to allergic sensitisation, and increase the accessibility of factors inducing BHR (114). Interestingly, anti-inflammatory corticosteroids not only reduce inflammation in the lung, but also may improve epithelial repair (115) and epithelial barrier function (116, 117), by upregulation of adhesion molecules. Thus by restoring the protective epithelial phenotype corticosteroids may also improve BHR.

Overall, the changes to the smooth muscle cells and airway epithelium may in combination with subepithelial extracellular matrix deposition, basement membrane thickening, increased numbers of activated fibroblasts, and vascular changes, all contribute to BHR. Some of these structural changes to the airway may not only contribute to BHR and asthma, but are also thought be compensating measures of the airway towards the increasing bronchoconstriction or environmental insults. Evidence of airway remodelling as an increase

in reticular membrane thickness was already visible in children before asthma was clinically diagnosed (118). In addition, in patients who were in complete asthma remission airway remodelling is retained, as evidenced by presence of a thickened reticular basement membrane (119). In an animal study, remodelling was even shown to be protective for airway hyperresponsiveness. Rats exposed to the model allergen ovalbumin initially developed remodelling, and an increase in airway hyperresponsiveness. After longer period of allergen exposure more extracellular matrix was deposited, the number of goblet cells and tissue eosinophils were still increased, but the difference in hyperresponsiveness had disappeared (120). Furthermore in asthmatic patients the increase in reticular basement membrane thickness positively correlated with methacholine PD20 in asthma patients (121). Altogether, the stiffening of the airway wall by ECM deposition and increase in RBM thickness may thereby protect for the increase in ASM-contractility and hyperresponsiveness (122).

Clearly, the recent research efforts have increased our understanding of the pathogenesis of asthma and BHR, and the possible functional consequences of airway inflammation and remodelling. Our increasing knowledge also gives insight into the extreme complexity of the disease. Therefore we carefully need to indentify the underlying mechanisms of inflammation, remodelling and the contribution and significance to BHR and asthma. In order to further understand the origins of asthma, first the genetic background of asthma and BHR has to be revealed. Genetic studies are able to identify novel asthma and BHR susceptibility genes and may provide new clues in dissecting the underlying mechanisms in asthma and BHR. Therefore, evidence for the genetic contribution to asthma, and the evidence for asthma genes will be reviewed below.

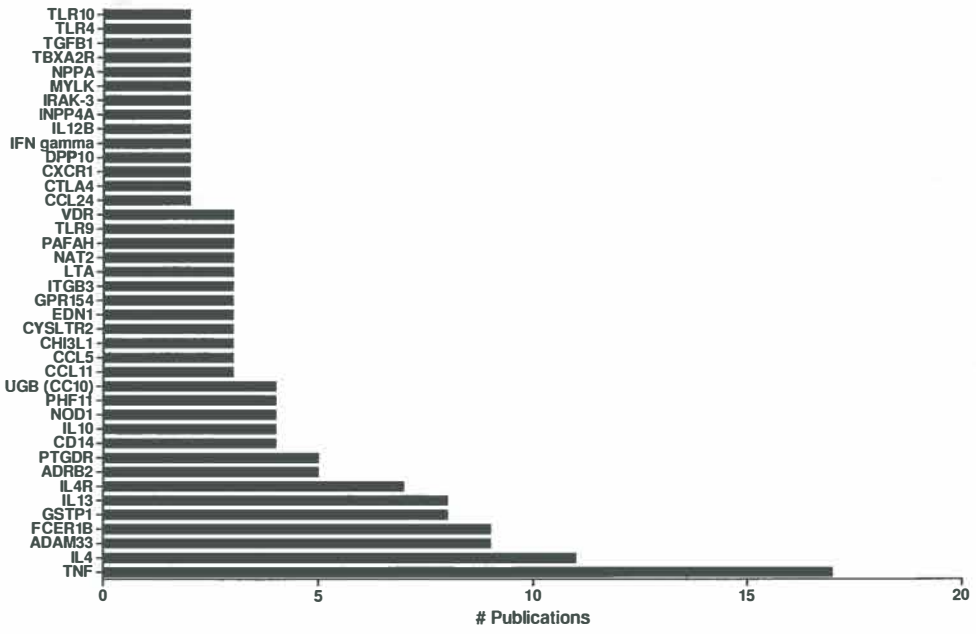
Genetic background of asthma

Twin studies have indicated that the susceptibility to develop asthma has a considerable heritable component (50-90%), but that environmental factors also contribute (123, 124). In order to identify the genomic regions which are co-inherited with asthma, several genome-wide linkage studies were conducted. These studies typically used a set of polymorphic short tandem repeats or micro-satellite markers to cover the genome, and aimed to identify the genomic regions that may contain asthma susceptibility genes. Regions on chromosomes

2q22-33, 5q31.1-33, 6p21.3, 11q13, 12q14.3-24.1, 13q14, 14q11.2-13 and 19q13 were identified to contain susceptibility genes for asthma and related traits like BHR, total IgE-levels and positive skin prick tests (125). To determine which genes are responsible for the linkage-signal identified at these chromosomes, classical candidate gene association studies were performed. Candidate genes were selected based on their known function and potential involvement in asthma pathogenesis (126). Chromosome 5 for example, a region identified by a Dutch linkage study (127), harbours several genes in immune pathways (IL-4, IL-13; 5q31) and bronchodilator response (β2-adrenergic receptor (ADRB2), 5q33) (128) that are associated with asthma and reported by many studies (Figure 2). In addition, single nucleotide polymorphisms (SNPs) of genes in well known pathways, such as the TLR-pathway, were shown to associate with asthma (129), but are reported by fewer studies (Figure 2). Altogether, a large number of candidate genes for asthma are identified using these candidate gene association studies (130) (see Figure 2).

Figure 2: Asthma candidate genes

Replicated asthma candidate genes are shown and sorted according to their number of publications. Adapted from Weiss, Raby and Rogers, Asthma genetics and genomics 2009, Current Opinion in Genetics & Development 2009 (130).



The advantages of candidate gene studies are that they are powerful studies in which novel gene asthma associations can be quickly identified using relatively small populations, while the required prior knowledge on the genes and pathways to investigate may induce a study bias and are disadvantageous. Furthermore, identified associated SNPs are not always replicated (77). Therefore, unbiased approaches are needed in order to reveal new genes for asthma. These approaches are chromosome or genome-wide screens, such as positional cloning and genome-wide association studies (GWAS). These screens employ a dense set of SNPs to cover a genomic region previously linked to asthma (positional cloning) or the complete genome (GWAS). Although these studies can identify previously unknown disease associated genes, they have lower power due to the number of tests that need to be performed, and thus require large (replication) populations (131). Still, these studies identified several novel susceptibility genes as summarized in Table I.

Table I – Asthma genes identified by Positional Cloning and Genome Wide Association Studies

Positional Cloning

Gene(s)	Expression / Proposed function	Chromosome	Study
ADAM33	Smooth muscle, embryonic mesenchymal cells, Epithelium	20p13	(132)
DPP10	Brain, Immune cells (peptidase of chemokines)	2q14	(133)
PHF11	Immune related tissues	13q14	(134)
NPSR1/GPRA/GPR154	A isoform predominantly in smooth muscle, B isoform predominantly in epithelium	7p14	(135)
HLA-G	Immune cells; epithelium	6p21	(136)
CYF1P2	Undifferentiated lymphocytes	5q33	(137)
SFRS8	Regulates splicing of CD45 of T-cells	12q24	(138)
PLAUR / UPAR	PBMCs, Epithelium	19q13	(139)
PCDH1	Brain, Epithelial cells in lung	5q31.3	(140)

Genome Wide Association Studies

Gene(s)	Expression / Proposed function	Chromosome	Study
ORMDL3/GSMDL	Endoplasmatic Reticulum of several cells GSDML: Epithelium	17q21	(141-144)
IL1RL1 / IL18R1	Range of immune cells, epithelium IL-33 receptor	2q12	(142-145)
WDR36 / TSLP	T-cells (activation protein) / Epithelium (cytokine)	5q22	(145, 146)
IL-33	Stored in a.o smooth muscle and epithelium	9p24	(145)
MYB	TF for hematopoietic stem and prog. cells	6q23	(145)
RAD50-IL13	Constitutively expressed (DNA repair - Interleukin)	5q31.1	(147)
HLA-DQ	Lymphocytes and several tissues	6p21.3	(142, 144, 146, 147)
HLA-DP	Lymphocytes and several tissues	6p33	(148)
PDE4D	cAMP signaling - Regulates a.o.ASM contractility	5q12	(149)
ADRA1B	human vascular smooth muscle cells	5q33	(150)
DPP10	Brain, Immune cells, rat bronch.epithelium Peptidase of chemokines	2q12.3-q14.2	(150)

PRNP	Brain, Immune cells (T and B-cells, DCs)	20pter-p12	(150)
SMAD3	TGFb signalling, several cell-types	15	(142)
IL2RB	T-cell differentiation (T_H1, T_H2, T_H17)	22	(142)
Near GATA3 (1Mb)	Regulator of T_H2-cell differentiation	10p14	(146)
Near IKZF4 or EOS (2kb)	Differentiation of T-reg cells	12q13	(146)
USP38-GAB1	Immune cells (cytokine signalling)	4q31	(146)
IL6R	Hepatocytes and several leukocytes	1q21.3	(143)
Near LRRC32	Activated Treg cells	11q13.5	(143)
PYHIN1 or IFIX	Leukocytes, lung tissues	1q23	(144)
ATPAF1	Range of cell-types, purinergic (ATP)-signaling	1p31-36	(151)
DENND1B	Natural killer cells and dendritic cells	1q31.3	(152)
PERLD1	GPI-anchors for T-cell stimulation	17q12	(153)
TLE4	B-cell differentiation	9q21	(154)

PBMCs = Primary Blood Mononuclear Cells; DC = Dendritic Cell; TF = Transcription factor; cAMP = cyclic Adenosine Mono Phosphate; ASM = airway smooth muscle; GPI = glycosylphophatidylinositol.

Classical candidate studies have identified association of many genes in immune pathways. Interestingly, many of the novel genes identified by positional cloning and GWAS are expressed at the interface of structural cells of the airway, such as the airway epithelium, and the innate or adaptive immune system. (*PLAUR, NPSR1, HLA-G, PCDH1, TSLP, IL1RL1, IL-33, SMAD3*). These results suggest that, besides the immune system the airway epithelium plays an important role in asthma pathogenesis, and that dysfunction of the epithelial cells is in part due to intrinsic heritable genetic differences. Moreover, release of inflammatory mediators from the airway epithelium, such as TSLP and IL-33, may subsequently activate the immune system, amongst others through the IL-33-receptor IL1RL1, leading to differentiation and activation of DCs and T_H2-cells, while IL-33 pathway activation in mast cells can result in degranulation of these cells, and thereby contribute to bronchial hyperresponsiveness (155). Also genes involved in non-allergic non-immune pathways may contribute to asthma pathogenesis. Recent functional studies on ADAM33 indicate involvement of smooth muscle and vascular remodelling pathways in asthma. In addition, the 17q.21 locus (*ORMDL3 / GSDML*) is specifically associated with childhood-onset asthma. *ORMDL3* encodes for a protein that localizes to the endoplasmatic reticulum (ER), its dysfunction is thought to induce cellular stress (156), and may therefore be a novel genetic cause of asthma (126).

Over time, more and more knowledge regarding the genetic background of asthma is being obtained. But a large number of discovered asthma genes have not been replicated and most genetic variants only explain a small proportion of the genetic heritability of asthma (130). The lack of replication of some asthma genes may be the result of a difference in the asthma-phenotype used in the different populations. Furthermore, as asthma is characterized

by an interplay of genetic and environmental factors (157), gene-gene and gene-environment interactions will also have to be determined. These gene-gene and gene-environment interactions may account for part of the "missing" heritability (158). Interestingly, gene-gene interaction analysis of genes annotated to the TLR-pathway and regulatory T-cell function, resulted in significant associations of combinations of SNPs with asthma or atopy, while the identified SNPs did not always display a significant association with asthma or atopy in a single-SNP association analysis (129, 159). These results suggest that indeed genetic effects may be missed when these gene-gene interactions are not taken into account.

Environmental factors may influence the risk for asthma development. Examples are: endotoxin exposure (160), either due to day-care attendance, or living in a farming environment (161); air-traffic related pollution, due to particulate matter (PM(2.5)), soot and nitrogen dioxide (NO_2)) (162); cigarette smoke exposure, either intrauterine or environmental (163); and exposure to allergens from house-dust mite, dogs, cats (163), and farm living (161). Interestingly, results from a recent study of day-care attendance suggest that subjects with one *TLR2*-genotype (T-allele) may be protected against atopic wheezing development at day-care, while carriers of the other *TLR2*-genotype (AA-alleles) may be protected for atopic wheezing development when not attending day-care. Thus different environments may cause opposing effects on atopic wheezing development with a difference in genotype (164), pointing towards the complexity of gene-environment interactions. Therefore it is important to not only characterize the disease phenotype, but also the environmental exposures in great detail, and test for gene-environment interactions in large populations in order to determine the effect of both genetic variation and the environment, as well as their interactions, on asthma development.

As the gene-gene and gene-environment interactions are extremely complex in asthma, it is similarly complex to understand how these statistical associations can be translated into altered biology in structural components of the lung, such as the airway epithelium. Therefore, functional studies are required, in which the gene function and the influence of environmental exposures on gene function is investigated, as well as the functional role of asthma associated SNPs in the gene, in order to further understand the role of newly identified susceptibility genes and its interaction with environmental exposures in asthma pathogenesis.

The Protocadherin gene family

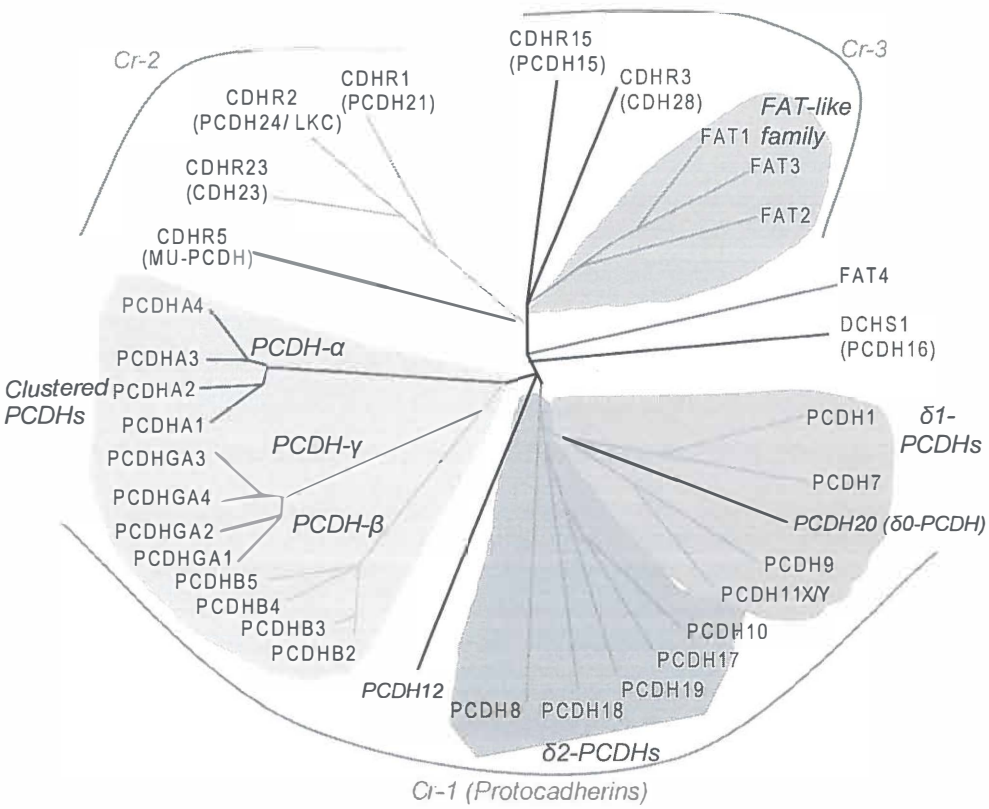
This thesis will focus on one of the novel susceptibility genes for BHR identified by positional cloning: Protocadherin-1 (*PCDH1*) ((140), Table I). Protocadherins are a subfamily of the cadherin superfamily of adhesion molecules, and were initially identified in brain tissue (165). The Protocadherin-subfamily currently consists of 65 members and thereby is the largest subfamily of the cadherin superfamily (166). The protocadherin family can be further subdivided in clustered (53 members, consisting of α, β, and γ-pcdh) and non-clustered protocadherins (12 members, consisting of δ1 and δ2-protocadherins) (Figure 3) (166). Most protocadherins show expression in the central nervous system, but some non-clustered Protocadherins also show expression in other organs, such as kidney and lung (167). *PCDH1* is a member of the δ1-subfamily of non-clustered protocadherin genes, which further consists of *PCDH7*, *PCDH9* and *PCDH11X/Y* (168). δ1-protocadherin gene-variants are associated with several diseases: *PCDH7* SNPs act as prognostic factors for the overall survival in early stage non-small-cell lung cancer (169) and a *PCDH11X* polymorphism associate with susceptibility to late-onset Alzheimer's disease (170). Furthermore PCDH9 protein expression was decreased in human cerebral glial tumors, which suggests that PCDH9 is an independent biological marker for survival of patients with gliomas (171).

δ1-protocadherins are characterized by the presence of seven extracellular cadherin repeats (ECs), and three evolutionary conserved motifs in their intracellular tail (CM1-3) (166). The protein has been implicated in tissue formation by cell-sorting (172), and displays weak adhesion activity (165). Classical cadherins like E-cadherin, typically mediate adhesion through homotypic engagement of the first EC-domains (EC1), which is dependent on calcium-ions and tryptophan residues (173). The adhesive properties of protocadherins are generally weaker than that of classical cadherins (165), as protocadherins do not contain the conserved tryptophan residues in their EC1-domains (174). Adhesion of protocadherins may form by other mechanisms such as binding of calcium-ions by polar amino acids at the N-terminus of the EC1 (175), or may even not be its main function. Most δ1-protocadherins encode multiple isoforms, including one with and one without conserved CM1-3 motifs (168). The presence of these evolutionarily conserved signalling motifs in the intracellular domains may point towards a function for δ1-protocadherins in intracellular signal transduction (168). Indeed, the adaptor molecule protein phosphatase 1 alpha (PP1α) was shown to bind to the CM3-domain, which indicates that at least one of the protocadherin isoforms participate in

intracellular signal transduction (176). Overall, protocadherins are involved in different diseases and may participate in cell-cell adhesion or intracellular signalling processes, but their functions remain to be elucidated.

Figure 3: Phylogenetic tree of the Protocadherin subfamily of adhesion molecules and related molecules

A phylogenetic tree is constructed based on the intracellular sequences of representative protocadherins and related molecules. The protocadherin subfamily can be categorized into clustered and non-clustered (δ1 and δ2-PCDHs, PCDH12, PCDH20) protocadherins, also referred to as cadherin-related 1 cluster (Cr-1). PCDH20 has high similarity with the extracellular cadherin domains (EC) of δ1-pcdhs, but has only a short intracellular tail and is therefore not considered a δ1-PCDH, but named δ0-PCDH (166). PCDH15, FAT, FAT-like, and Dachsous (DCHS) cadherin molecules are large molecules and contain 11 up to 34 EC-domains, while CDHR5/Mu-PCDH only contains 4 EC-domains. CDHR1/PCDH21, CDHR2 /PCDH24, CDHR5/Mu-PCDH, and PCDH15 are very distant from protocadherins and are therefore named Cadherin-related (CDHR) (166), and clustered in the cadherin-related 2 (Cr-2) or 3 (Cr-3) clusters, together with CDHR23/CDH23 (Cr-2), and CDHR3/CDH28, and FAT-like molecules (Cr-3). Adapted from Redies et al., 2005 (168); and Hulpiau and Van Roy, 2009 (166).



Aims of this thesis

In this thesis we aim to investigate the role of Protocadherin-1 in asthma pathogenesis. As protocadherins are involved in cell-cell adhesion, and the epithelial barrier in asthma is compromised, we hypothesize that PCDH1 dysfunction leads to increased susceptibility of the bronchial epithelium to environmental insults, like house-dust mite allergen and environmental tobacco smoke exposure, which leads to a decreased epithelial integrity and attenuated repair responses, which contribute to asthma and BHR development. Therefore, we investigate the genetics, expression, regulation and function of Protocadherin-1. In chapter 2, we describe the identification of this gene as a novel gene for BHR and asthma. Previously the chromosomal region 5q31-33 was linked to BHR (127). We aimed to identify which gene on 5q31-33 contributes to the observed linkage signal, and describe the genetic variation of this gene in the Dutch population and its association with BHR and asthma. We then replicate these findings in different populations from the Netherlands, the United Kingdom and the United States. We subsequently investigate PCDH1 mRNA and protein expression in the airways.

In chapter 3, we aim to identify whether *PCDH1* polymorphisms that were previously found to be associated with BHR and asthma, also associate with eczema. As PCDH1 is expressed in skin epithelium as well (177), we aimed to investigate whether asthma and eczema have a common genetic background that could in part be attributed to variation in *PCDH1*.

In chapter 4 we aim to describe PCDH1 mRNA and protein isoforms and their expression levels in freshly isolated and cultured Primary Bronchial Epithelial Cells (PBECs) of asthma patients. Furthermore we aimed to investigate whether PCDH1 expression was regulated by airway epithelial mucociliary differentiation ex vivo.

In chapter 5, we aimed to test whether *Pcdh1* expression *in vivo* is regulated by relevant environmental exposures. To this end, we first established the expression levels of Pcdh1 in mouse lung under basal conditions. Next, we assessed the regulation of Pcdh1 expression by environmental challenges that induce airway hyperresponsiveness, such as house dust mite and cigarette smoke exposure.

In chapter 6 we aimed to functionally characterize Pcdh1 proteins, by investigating the subcellular localization of the different PCDH1 protein isoforms in bronchial epithelial cells. Furthermore we tested whether interactions of the PCDH1 protein isoforms with a candidate interacting protein SMAD3 (178), that was recently also identified as a novel asthma gene and is expressed in airway epithelial cells (142).

Finally, in chapter 7, the previous chapters are discussed and suggestions for future research are given.

REFERENCES

1. Masoli M, Fabian D, Holt S, Beasley R , Global Initiative for Asthma (GINA) Program. The global burden of asthma: Executive summary of the GINA dissemination committee report. Allergy. 2004;59(5):469-78.
2. Sears MR, Greene JM, Willan AR, Wiecek EM, Taylor DR, Flannery EM, et al. A longitudinal, population-based, cohort study of childhood asthma followed to adulthood. N Engl J Med. 2003;349(15):1414-22.
3. Lemanske RF,Jr, Busse WW. Asthma: Clinical expression and molecular mechanisms. J Allergy Clin Immunol. 2010;125(2 Suppl 2):S95-102.
4. Spahn JD, Covar R. Clinical assessment of asthma progression in children and adults. J Allergy Clin Immunol. 2008;121(3):548,57; quiz 558-9.
5. Savenije OE, Granell R, Caudri D, Koppelman GH, Smit HA, Wijga A, et al. Comparison of childhood wheezing phenotypes in 2 birth cohorts: ALSPAC and PIAMA. J Allergy Clin Immunol. 2011; 127(6):1505,12.e14.
6. Martinez FD. Development of wheezing disorders and asthma in preschool children. Pediatrics. 2002;109(2 Suppl):362-7.
7. van den Berge M, Heijink HI, van Oosterhout AJ, Postma DS. The role of female sex hormones in the development and severity of allergic and non-allergic asthma. Clin Exp Allergy. 2009;39(10):1477-81.
8. Http://www.ginasthma.org/pdf/GINA_Report_2010.pdf [Internet].
9. Jansen DF, Timens W, Kraan J, Rijcken B, Postma DS. (A)symptomatic bronchial hyper-responsiveness and asthma. Respir Med. 1997;91(3):121-34.
10. Rage E, Siroux V, Le Moual N, Pin I, Kauffmann F. Re: Are asymptomatic airway hyperresponsiveness and allergy risk factors for asthma? A longitudinal study. Eur Respir J. 2009;33(1):218-9.
11. Van Den Berge M, Meijer RJ, Kerstjens HA, de Reus DM, Koeter GH, Kauffman HF, et al. PC(20) adenosine 5'-monophosphate is more closely associated with airway inflammation in asthma than PC(20) methacholine. Am J Respir Crit Care Med. 2001;163(7):1546-50.
12. van den Berge M, Kerstjens HA, Meijer RJ, de Reus DM, Koeter GH, Kauffman HF, et al. Corticosteroid-induced improvement in the PC20 of adenosine monophosphate is more closely associated with reduction in airway inflammation than improvement in the PC20 of methacholine. Am J Respir Crit Care Med. 2001; 164(7):1127-32.
13. Busse WW. The relationship of airway hyperresponsiveness and airway inflammation: Airway hyperresponsiveness in asthma: Its measurement and clinical significance. Chest. 2010;138(2 Suppl):4S-10S.
14. Svensson M, Kaye PM. Stromal-cell regulation of dendritic-cell differentiation and function. Trends Immunol. 2006;27(12):580-7.
15. Hammad H, Lambrecht BN. Dendritic cells and airway epithelial cells at the interface between innate and adaptive immune responses. Allergy. 2011;66(5):579-87.
16. Palomares O, Yaman G, Azkur AK, Akkoc T, Akdis M, Akdis CA. Role of treg in immune regulation of allergic diseases. Eur J Immunol. 2010;40(5):1232-40.
17. Oliphant CJ, Barlow JL, McKenzie AN. Insights into the initiation of type 2 immune responses. Immunology. 2011;134(4):378-85.
18. Li H, Rostami A. IL-9: Basic biology, signaling pathways in CD4+ T cells and implications for autoimmunity. J Neuroimmune Pharmacol. 2010;5(2):198-209.
19. Minai-Fleminger Y, Levi-Schaffer F. Mast cells and eosinophils: The two key effector cells in allergic inflammation. Inflamm Res. 2009;58(10):631-8.
20. O'Byrne P. Asthma pathogenesis and allergen-induced late responses. J Allergy Clin Immunol. 1998; 102(5):S85-9.
21. Weaver CT, Hatton RD, Mangan PR, Harrington LE. IL-17 family cytokines and the expanding diversity of effector T cell lineages. Annu Rev Immunol. 2007;25:821-52.
22. Robinson DS. The role of the T cell in asthma. J Allergy Clin Immunol. 2010;126(6):1081-91.
23. Enk AH. Dendritic cells in tolerance induction. Immunol Lett. 2005;99(1):8-11.
24. Venuprasad K, Kong YC, Farrar MA. Control of Th2-mediated inflammation by regulatory T cells. Am J Pathol. 2010;177(2):525-31.

25. Lloyd CM, Hessel EM. Functions of T cells in asthma: More than just T(H)2 cells. Nat Rev Immunol. 2010;10(12):838-48.
26. Durrant DM, Metzger DW. Emerging roles of T helper subsets in the pathogenesis of asthma. Immunol Invest. 2010;39(4-5):526-49.
27. Laitinen A, Laitinen LA. Airway morphology: Epithelium/basement membrane. Am J Respir Crit Care Med. 1994;150(5 Pt 2):S14-7.
28. Westergren-Thorsson G, Larsen K, Nihlberg K, Andersson-Sjoland A, Hallgren O, Marko-Varga G, et al. Pathological airway remodelling in inflammation. Clin Respir J. 2010;4 Suppl 1:1-8.
29. Bergeron C, Boulet LP. Structural changes in airway diseases: Characteristics, mechanisms, consequences, and pharmacologic modulation. Chest. 2006;129(4):1068-87.
30. Nawijn MC, Hackett TL, Postma DS, van Oosterhout AJ, Heijink IH. E-cadherin: Gatekeeper of airway mucosa and allergic sensitization. Trends Immunol. 2011;32(6):248-55.
31. Wiszniewski L, Sanz J, Scerri I, Gasparotto E, Dudez T, Lacroix JS, et al. Functional expression of connexin30 and connexin31 in the polarized human airway epithelium. Differentiation. 2007;75(5):382-92.
32. Crystal RG, Randell SH, Engelhardt JF, Voynow J, Sunday ME. Airway epithelial cells: Current concepts and challenges. Proc Am Thorac Soc. 2008;5(7):772-7.
33. Montefort S, Baker J, Roche WR, Holgate ST. The distribution of adhesive mechanisms in the normal bronchial epithelium. Eur Respir J. 1993;6(9):1257-63.
34. Giepmans BN, van Ijzendoorn SC. Epithelial cell-cell junctions and plasma membrane domains. Biochim Biophys Acta. 2009;1788(4):820-31.
35. Hackett NR, Shaykhiev R, Walters MS, Wang R, Zwick RK, Ferris B, et al. The human airway epithelial basal cell transcriptome. PLoS One. 2011;6(5):e18378.
36. Knight DA, Holgate ST. The airway epithelium: Structural and functional properties in health and disease. Respirology. 2003;8(4):432-46.
37. Jeffery PK, Li D. Airway mucosa: Secretory cells, mucus and mucin genes. Eur Respir J. 1997;10(7):1655-62.
38. Young LR, Brody AS, Inge TH, Acton JD, Bokulic RE, Langston C, et al. Neuroendocrine cell distribution and frequency distinguish neuroendocrine cell hyperplasia of infancy from other pulmonary disorders. Chest. 2011;139(5):1060-71.
39. Linnoila RI. Functional facets of the pulmonary neuroendocrine system. Lab Invest. 2006;86(5):425-44.
40. Muir A, Soong G, Sokol S, Reddy B, Gomez MI, Van Heeckeren A, et al. Toll-like receptors in normal and cystic fibrosis airway epithelial cells. Am J Respir Cell Mol Biol. 2004;30(6):777-83.
41. Vareille M, Kieninger E, Edwards MR, Regamey N. The airway epithelium: Soldier in the fight against respiratory viruses. Clin Microbiol Rev. 2011;24(1):210-29.
42. Chen K, Xiang Y, Yao X, Liu Y, Gong W, Yoshimura T, et al. The active contribution of toll-like receptors to allergic airway inflammation. Int Immunopharmacol. 2011;11(10):1391-8.
43. Kato A, Favoreto S,Jr, Avila PC, Schleimer RP. TLR3- and Th2 cytokine-dependent production of thymic stromal lymphopoietin in human airway epithelial cells. J Immunol. 2007;179(2):1080-7.
44. Chen K, Huang J, Gong W, Iribarren P, Dunlop NM, Wang JM. Toll-like receptors in inflammation, infection and cancer. Int Immunopharmacol. 2007;7(10):1271-85.
45. Bianchi ME. DAMPs, PAMPs and alarmins: All we need to know about danger. J Leukoc Biol. 2007; 81(1):1-5.
46. Ferhani N, Letuve S, Kozhich A, Thibaudeau O, Grandsaigne M, Maret M, et al. Expression of high-mobility group box 1 and of receptor for advanced glycation end products in chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2010;181(9):917-27.
47. Moussion C, Ortega N, Girard JP. The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: A novel 'alarmin'? PLoS One. 2008;3(10):e3331.
48. Kono H, Rock KL. How dying cells alert the immune system to danger. Nat Rev Immunol. 2008;8(4):279-89.
49. Holgate ST, Davies DE, Puddicombe S, Richter A, Lackie P, Lordan J, et al. Mechanisms of airway epithelial damage: Epithelial-mesenchymal interactions in the pathogenesis of asthma. Eur Respir J Suppl. 2003; 44:24s-9s.
50. Morishima Y, Nomura A, Uchida Y, Noguchi Y, Sakamoto T, Ishii Y, et al. Triggering the induction of myofibroblast and fibrogenesis by airway epithelial shedding. Am J Respir Cell Mol Biol. 2001;24(1):1-11.
51. Warburton D, Schwarz M, Tefft D, Flores-Delgado G, Anderson KD, Cardoso WV. The molecular basis of lung morphogenesis. Mech Dev. 2000;92(1):55-81.
52. Gerthoffer WT. Migration of airway smooth muscle cells. Proc Am Thorac Soc. 2008;5(1):97-105.
53. Marston SB, Smith CW. The thin filaments of smooth muscles. J Muscle Res Cell Motil. 1985;6(6):669-708.

54. Mitzner W. Airway smooth muscle: The appendix of the lung. Am J Respir Crit Care Med. 2004; 169(7):787-90.
55. Volckaert T, Dill E, Campbell A, Tiozzo C, Majka S, Bellusci S, et al. Parabronchial smooth muscle constitutes an airway epithelial stem cell niche in the mouse lung after injury. J Clin Invest. 2011;121(11):4409-19.
56. Fahy JV. Eosinophilic and neutrophilic inflammation in asthma: Insights from clinical studies. Proc Am Thorac Soc. 2009;6(3):256-9.
57. Al-Muhsen S, Johnson JR, Hamid Q. Remodeling in asthma. J Allergy Clin Immunol. 2011;128(3):451,62; quiz 463-4.
58. Bergeron C, Al-Ramli W, Hamid Q. Remodeling in asthma. Proc Am Thorac Soc. 2009;6(3):301-5.
59. Proud D, Leigh R. Epithelial cells and airway diseases. Immunol Rev. 2011;242(1):186-204.
60. Tsartsali L, Hislop AA, McKay K, James AL, Elliot J, Zhu J, et al. Development of the bronchial epithelial reticular basement membrane: Relationship to epithelial height and age. Thorax. 2011;66(4):280-5.
61. Zanini A, Chetta A, Imperatori AS, Spanevello A, Olivieri D. The role of the bronchial microvasculature in the airway remodelling in asthma and COPD. Respir Res. 2010;29;11:132.
62. Boulet LP, Laviolette M, Turcotte H, Cartier A, Dugas M, Malo JL, et al. Bronchial subepithelial fibrosis correlates with airway responsiveness to methacholine. Chest. 1997;112(1):45-52.
63. Yamada Y, Yoshihara S, Arisaka O. Creola bodies in wheezing infants predict the development of asthma. Pediatr Allergy Immunol. 2004;15(2):159-62.
64. Ordonez C, Ferrando R, Hyde DM, Wong HH, Fahy JV. Epithelial desquamation in asthma: Artifact or pathology? Am J Respir Crit Care Med. 2000;162(6):2324-9.
65. Ordonez CL, Fahy JV. Epithelial desquamation in asthma. Am J Respir Crit Care Med. 2001;164(10 Pt 1):1997.
66. de Boer WI, Sharma HS, Baelemans SM, Hoogsteden HC, Lambrecht BN, Braunstahl GJ. Altered expression of epithelial junctional proteins in atopic asthma: Possible role in inflammation. Can J Physiol Pharmacol. 2008;86(3):105-12.
67. Xiao C, Puddicombe SM, Field S, Haywood J, Broughton-Head V, Puxeddu I, et al. Defective epithelial barrier function in asthma. J Allergy Clin Immunol. 2011 Jul 11.
68. Hackett TL, Singhera GK, Shaheen F, Hayden P, Jackson GR, Hegele RG, et al. Intrinsic phenotypic differences of asthmatic epithelium and its inflammatory responses to RSV and air pollution. Am J Respir Cell Mol Biol. 2011 Jun 3.
69. Xiao C, Puddicombe SM, Field S, Haywood J, Broughton-Head V, Puxeddu I, et al. Defective epithelial barrier function in asthma. J Allergy Clin Immunol. 2011;128(3):549,56.e1-12.
70. Thomas B, Rutman A, Hirst RA, Haldar P, Wardlaw AJ, Bankart J, et al. Ciliary dysfunction and ultrastructural abnormalities are features of severe asthma. J Allergy Clin Immunol. 2010;126(4):722,729.e2.
71. Ordonez CL, Khashayar R, Wong HH, Ferrando R, Wu R, Hyde DM, et al. Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression. Am J Respir Crit Care Med. 2001;163(2):517-23.
72. DUNNILL MS. The pathology of asthma, with special reference to changes in the bronchial mucosa. J Clin Pathol. 1960;13:27-33.
73. Kicic A, Sutanto EN, Stevens PT, Knight DA, Stick SM. Intrinsic biochemical and functional differences in bronchial epithelial cells of children with asthma. Am J Respir Crit Care Med. 2006;174(10):1110-8.
74. Uller L, Leino M, Bedke N, Sammut D, Green B, Lau L, et al. Double-stranded RNA induces disproportionate expression of thymic stromal lymphopoietin versus interferon-beta in bronchial epithelial cells from donors with asthma. Thorax. 2010;65(7):626-32.
75. Vignola AM, Chanez P, Chiappara G, Merendino A, Pace E, Rizzo A, et al. Transforming growth factor-beta expression in mucosal biopsies in asthma and chronic bronchitis. Am J Respir Crit Care Med. 1997;156(2 Pt 1):591-9.
76. Puddicombe SM, Polosa R, Richter A, Krishna MT, Howarth PH, Holgate ST, et al. Involvement of the epidermal growth factor receptor in epithelial repair in asthma. FASEB J. 2000;14(10):1362-74.
77. Howard TD, Meyers DA, Bleecker ER. Mapping susceptibility genes for asthma and allergy. J Allergy Clin Immunol. 2000;105(2 Pt 2):S477-81.
78. Boxall C, Holgate ST, Davies DE. The contribution of transforming growth factor-beta and epidermal growth factor signalling to airway remodelling in chronic asthma. Eur Respir J. 2006;27(1):208-29.
79. Davies DE. The role of the epithelium in airway remodeling in asthma. Proc Am Thorac Soc. 2009;6(8):678-82.
80. Holgate ST. Epithelium dysfunction in asthma. J Allergy Clin Immunol. 2007;120(6):1233,44; quiz 1245-6.
81. Ebina M, Takahashi T, Chiba T, Motomiya M. Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma. A 3-D morphometric study. Am Rev Respir Dis. 1993;148(3):720-6.

82. Benayoun L, Druilhe A, Dombret MC, Aubier M, Pretolani M. Airway structural alterations selectively associated with severe asthma. Am J Respir Crit Care Med. 2003;167(10):1360-8.
83. Woodruff PG, Dolganov GM, Ferrando RE, Donnelly S, Hays SR, Solberg OD, et al. Hyperplasia of smooth muscle in mild to moderate asthma without changes in cell size or gene expression. Am J Respir Crit Care Med. 2004;169(9):1001-6.
84. Johnson PR, Burgess JK, Underwood PA, Au W, Poniris MH, Tamm M, et al. Extracellular matrix proteins modulate asthmatic airway smooth muscle cell proliferation via an autocrine mechanism. J Allergy Clin Immunol. 2004:113(4):690-6.
85. Araujo BB, Dolhnikoff M, Silva LF, Elliot J, Lindeman JH, Ferreira DS, et al. Extracellular matrix components and regulators in the airway smooth muscle in asthma. Eur Respir J. 2008;32(1):61-9.
86. Bara I, Ozier A, Tunon de Lara JM, Marthan R, Berger P. Pathophysiology of bronchial smooth muscle remodelling in asthma. Eur Respir J. 2010;36(5):1174-84.
87. Alkhouri H, Hollins F, Moir LM, Brightling CE, Armour CL, Hughes JM. Human lung mast cells modulate the functions of airway smooth muscle cells in asthma. Allergy. 2011;66(9):1231-41.
88. Goncharova EA, Billington CK, Irani C, Vorotnikov AV, Tkachuk VA, Penn RB, et al. Cyclic AMP-mobilizing agents and glucocorticoids modulate human smooth muscle cell migration. Am J Respir Cell Mol Biol. 2003;29(1):19-27.
89. Sutcliffe A, Hollins F, Gomez E, Saunders R, Doe C, Cooke M, et al. Increased nicotinamide adenine dinucleotide phosphate oxidase 4 expression mediates intrinsic airway smooth muscle hypercontractility in asthma. Am J Respir Crit Care Med. 2012;185(3):267-74.
90. Ma X, Cheng Z, Kong H, Wang Y, Unruh H, Stephens NL, et al. Changes in biophysical and biochemical properties of single bronchial smooth muscle cells from asthmatic subjects. Am J Physiol Lung Cell Mol Physiol. 2002;283(6):L1181-9.
91. Leguillette R, Lauzon AM. Molecular mechanics of smooth muscle contractile proteins in airway hyperresponsiveness and asthma. Proc Am Thorac Soc. 2008;5(1):40-6.
92. Kariyawasam HH, Aizen M, Barkans J, Robinson DS, Kay AB. Remodeling and airway hyperresponsiveness but not cellular inflammation persist after allergen challenge in asthma. Am J Respir Crit Care Med. 2007;175(9):896-904.
93. Sont JK, Willems LN, Bel EH, van Krieken JH, Vandenbroucke JP, Sterk PJ. Clinical control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide to long-term treatment. the AMPUL study group. Am J Respir Crit Care Med. 1999;159(4 Pt 1):1043-51.
94. Cockcroft DW, Davis BE. Mechanisms of airway hyperresponsiveness. J Allergy Clin Immunol. 2006; 118(3):551,9; quiz 560-1.
95. Durham SR, Craddock CF, Cookson WO, Benson MK. Increases in airway responsiveness to histamine precede allergen-induced late asthmatic responses. J Allergy Clin Immunol. 1988;82(5 Pt 1):764-70.
96. Aalbers R, Kauffman HF, Koeter GH, Postma DS, De Vries K, De Monchy JG. Dissimilarity in methacholine and adenosine 5'-monophosphate responsiveness 3 and 24 h after allergen challenge. Am Rev Respir Dis. 1991;144(2):352-7.
97. Boulet LP, Turcotte H, Laviolette M, Naud F, Bernier MC, Martel S, et al. Airway hyperresponsiveness, inflammation, and subepithelial collagen deposition in recently diagnosed versus long-standing mild asthma. influence of inhaled corticosteroids. Am J Respir Crit Care Med. 2000;162(4 Pt 1):1308-13.
98. Olivieri D, Chetta A, Del Donno M, Bertorelli G, Casalini A, Pesci A, et al. Effect of short-term treatment with low-dose inhaled fluticasone propionate on airway inflammation and remodeling in mild asthma: A placebo-controlled study. Am J Respir Crit Care Med. 1997;155(6):1864-71.
99. van Rensen EL, Straathof KC, Veselic-Charvat MA, Zwinderman AH, Bel EH, Sterk PJ. Effect of inhaled steroids on airway hyperresponsiveness, sputum eosinophils, and exhaled nitric oxide levels in patients with asthma. Thorax. 1999;54(5):403-8.
100. Kerstjens HA, Brand PL, Hughes MD, Robinson NJ, Postma DS, Sluiter HJ, et al. A comparison of bronchodilator therapy with or without inhaled corticosteroid therapy for obstructive airways disease. dutch chronic non-specific lung disease study group. N Engl J Med. 1992;327(20):1413-9.
101. Kraan J, Koeter GH, vd Mark TW, Sluiter HJ, de Vries K. Changes in bronchial hyperreactivity induced by 4 weeks of treatment with antiasthmatic drugs in patients with allergic asthma: A comparison between budesonide and terbutaline. J Allergy Clin Immunol. 1985;76(4):628-36.
102. Sawaguchi M, Tanaka S, Nakatani Y, Harada Y, Mukai K, Matsunaga Y, et al. Role of mast cells and basophils in IgE responses and in allergic airway hyperresponsiveness. J Immunol. 2012;188(4):1809-18.
103. Kariyawasam HH, Robinson DS. The role of eosinophils in airway tissue remodelling in asthma. Curr Opin Immunol. 2007;19(6):681-6.
104. Bradding P. The role of the mast cell in asthma: A reassessment. Curr Opin Allergy Clin Immunol. 2003; 3(1):45-50.

105. Flood-Page P, Swenson C, Faiferman I, Matthews J, Williams M, Brannick L, et al. A study to evaluate safety and efficacy of mepolizumab in patients with moderate persistent asthma. Am J Respir Crit Care Med. 2007;176(11):1062-71.
106. Nair P, Pizzichini MM, Kjarsgaard M, Inman MD, Efthimiadis A, Pizzichini E, et al. Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. N Engl J Med. 2009;360(10):985-93.
107. Lambert RK, Wiggs BR, Kuwano K, Hogg JC, Pare PD. Functional significance of increased airway smooth muscle in asthma and COPD. J Appl Physiol. 1993;74(6):2771-81.
108. Blacquiere MJ, Timens W, Melgert BN, Geerlings M, Postma DS, Hylkema MN. Maternal smoking during pregnancy induces airway remodelling in mice offspring. Eur Respir J. 2009;33(5):1133-40.
109. Siddiqui S, Mistry V, Doe C, Roach K, Morgan A, Wardlaw A, et al. Airway hyperresponsiveness is dissociated from airway wall structural remodeling. J Allergy Clin Immunol. 2008;122(2):335,41, 341.e1-3.
110. Carroll NG, Mutavdzic S, James AL. Distribution and degranulation of airway mast cells in normal and asthmatic subjects. Eur Respir J. 2002;19(5):879-85.
111. Tsurikisawa N, Oshikata C, Tsuburai T, Saito H, Sekiya K, Tanimoto H, et al. Bronchial hyperresponsiveness to histamine correlates with airway remodelling in adults with asthma. Respir Med. 2010;104(9):1271-7.
112. Amin K, Ekberg-Jansson A, Lofdahl CG, Venge P. Relationship between inflammatory cells and structural changes in the lungs of asymptomatic and never smokers: A biopsy study. Thorax. 2003;58(2):135-42.
113. Ohashi Y, Motojima S, Fukuda T, Makino S. Airway hyperresponsiveness, increased intracellular spaces of bronchial epithelium, and increased infiltration of eosinophils and lymphocytes in bronchial mucosa in asthma. Am Rev Respir Dis. 1992;145(6):1469-76.
114. Sparrow MP, Omari TI, Mitchell HW. The epithelial barrier and airway responsiveness. Can J Physiol Pharmacol. 1995;73(2):180-90.
115. Wadsworth SJ, Nijmeh HS, Hall IP. Glucocorticoids increase repair potential in a novel in vitro human airway epithelial wounding model. J Clin Immunol. 2006;26(4):376-87.
116. Sekiyama A, Gon Y, Terakado M, Takeshita I, Kozu Y, Maruoka S, et al. Glucocorticoids enhance airway epithelial barrier integrity. Int Immunopharmacol. 2011 Dec 27.
117. Laitinen LA, Laitinen A. Modulation of bronchial inflammation: Corticosteroids and other therapeutic agents. Am J Respir Crit Care Med. 1994;150(5 Pt 2):S87-90.
118. Pohunek P, Warner JO, Turzikova J, Kudrmann J, Roche WR. Markers of eosinophilic inflammation and tissue re-modelling in children before clinically diagnosed bronchial asthma. Pediatr Allergy Immunol. 2005;16(1):43-51.
119. Broekema M, Timens W, Vonk JM, Volbeda F, Lodewijk ME, Hylkema MN, et al. Persisting remodeling and less airway wall eosinophil activation in complete remission of asthma. Am J Respir Crit Care Med. 2011;183(3):310-6.
120. Palmans E, Kips JC, Pauwels RA. Prolonged allergen exposure induces structural airway changes in sensitized rats. Am J Respir Crit Care Med. 2000;161(2 Pt 1):627-35.
121. Milanese M, Crimi E, Scordamaglia A, Riccio A, Pellegrino R, Canonica GW, et al. On the functional consequences of bronchial basement membrane thickening. J Appl Physiol. 2001;91(3):1035-40.
122. McParland BE, Macklem PT, Pare PD. Airway wall remodeling: Friend or foe? J Appl Physiol. 2003;95(1):426-34.
123. van Beijsterveldt CE, Boomsma DI. Genetics of parentally reported asthma, eczema and rhinitis in 5-yr-old twins. Eur Respir J. 2007;29(3):516-21.
124. Thomsen SF, van der Sluis S, Kyvik KO, Skytthe A, Backer V. Estimates of asthma heritability in a large twin sample. Clin Exp Allergy. 2010;40(7):1054-61.
125. Denham S, Koppelman GH, Blakey J, Wjst M, Ferreira MA, Hall IP, et al. Meta-analysis of genome-wide linkage studies of asthma and related traits. Respir Res. 2008;9:38.
126. Ober C, Yao TC. The genetics of asthma and allergic disease: A 21st century perspective. Immunol Rev. 2011;242(1):10-30.
127. Postma DS, Bleecker ER, Amelung PJ, Holroyd KJ, Xu J, Panhuysen CI, et al. Genetic susceptibility to asthma--bronchial hyperresponsiveness coinherited with a major gene for atopy. N Engl J Med. 1995;333(14):894-900.
128. Cookson WO. Asthma genetics. Chest. 2002;121(3 Suppl):7S-13S.
129. Reijmerink NE, Bottema RW, Kerkhof M, Gerritsen J, Stelma FF, Thijs C, et al. TLR-related pathway analysis: Novel gene-gene interactions in the development of asthma and atopy. Allergy. 2010;65(2):199-207.
130. Weiss ST, Raby BA, Rogers A. Asthma genetics and genomics 2009. Curr Opin Genet Dev. 2009; 19(3):279-82.
131. Vercelli D. Discovering susceptibility genes for asthma and allergy. Nat Rev Immunol. 2008;8(3):169-82.

132. Van Eerdewegh P, Little RD, Dupuis J, Del Mastro RG, Falls K, Simon J, et al. Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness. Nature. 2002;418(6896):426-30.
133. Allen M, Heinzmann A, Noguchi E, Abecasis G, Broxholme J, Ponting CP, et al. Positional cloning of a novel gene influencing asthma from chromosome 2q14. Nat Genet. 2003;35(3):258-63.
134. Zhang Y, Leaves NI, Anderson GG, Ponting CP, Broxholme J, Holt R, et al. Positional cloning of a quantitative trait locus on chromosome 13q14 that influences immunoglobulin E levels and asthma. Nat Genet. 2003;34(2):181-6.
135. Laitinen T, Polvi A, Rydman P, Vendelin J, Pulkkinen V, Salmikangas P, et al. Characterization of a common susceptibility locus for asthma-related traits. Science. 2004;304(5668):300-4.
136. Nicolae D, Cox NJ, Lester LA, Schneider D, Tan Z, Billstrand C, et al. Fine mapping and positional candidate studies identify HLA-G as an asthma susceptibility gene on chromosome 6p21. Am J Hum Genet. 2005;76(2):349-57.
137. Noguchi E, Yokouchi Y, Zhang J, Shibuya K, Shibuya A, Bannai M, et al. Positional identification of an asthma susceptibility gene on human chromosome 5q33. Am J Respir Crit Care Med. 2005;172(2):183-8.
138. Brasch-Andersen C, Tan Q, Borglum AD, Haagerup A, Larsen TR, Vestbo J, et al. Significant linkage to chromosome 12q24.32-q24.33 and identification of SFRS8 as a possible asthma susceptibility gene. Thorax. 2006;61(10):874-9.
139. Barton SJ, Koppelman GH, Vonk JM, Browning CA, Nolte IM, Stewart CE, et al. PLAUR polymorphisms are associated with asthma, PLAUR levels, and lung function decline. J Allergy Clin Immunol. 2009;123(6):1391,400.e17.
140. Koppelman GH, Meyers DA, Howard TD, Zheng SL, Hawkins GA, Ampleford EJ, et al. Identification of PCDH1 as a novel susceptibility gene for bronchial hyperresponsiveness. Am J Respir Crit Care Med. 2009;180(10):929-35.
141. Moffatt MF, Kabesch M, Liang L, Dixon AL, Strachan D, Heath S, et al. Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. Nature. 2007;448(7152):470-3.
142. Moffatt MF, Gut IG, Demenais F, Strachan DP, Bouzigon E, Heath S, et al. A large-scale, consortium-based genomewide association study of asthma. N Engl J Med. 2010;363(13):1211-21.
143. Ferreira MA, Matheson MC, Duffy DL, Marks GB, Hui J, Le Souef P, et al. Identification of IL6R and chromosome 11q13.5 as risk loci for asthma. Lancet. 2011;378(9795):1006-14.
144. Torgerson DG, Ampleford EJ, Chiu GY, Gauderman WJ, Gignoux CR, Graves PE, et al. Meta-analysis of genome-wide association studies of asthma in ethnically diverse north american populations. Nat Genet. 2011;43(9):887-92.
145. Gudbjartsson DF, Bjornsdottir US, Halapi E, Helgadottir A, Sulem P, Jonsdottir GM, et al. Sequence variants affecting eosinophil numbers associate with asthma and myocardial infarction. Nat Genet. 2009; 41(3):342-7.
146. Hirota T, Takahashi A, Kubo M, Tsunoda T, Tomita K, Doi S, et al. Genome-wide association study identifies three new susceptibility loci for adult asthma in the japanese population. Nat Genet. 2011;43(9):893-6.
147. Li X, Howard TD, Zheng SL, Haselkorn T, Peters SP, Meyers DA, et al. Genome-wide association study of asthma identifies RAD50-IL13 and HLA-DR/DQ regions. J Allergy Clin Immunol. 2010;125(2):328,335.e11.
148. Noguchi E, Sakamoto H, Hirota T, Ochiai K, Imoto Y, Sakashita M, et al. Genome-wide association study identifies HLA-DP as a susceptibility gene for pediatric asthma in asian populations. PLoS Genet. 2011; 7(7):e1002170.
149. Himes BE, Hunninghake GM, Baurley JW, Rafaels NM, Sleiman P, Strachan DP, et al. Genome-wide association analysis identifies PDE4D as an asthma-susceptibility gene. Am J Hum Genet. 2009;84(5):581-93.
150. Mathias RA, Grant AV, Rafaels N, Hand T, Gao L, Vergara C, et al. A genome-wide association study on african-ancestry populations for asthma. J Allergy Clin Immunol. 2010;125(2):336,346.e4.
151. Schauberger EM, Ewart SL, Arshad SH, Huebner M, Karmaus W, Holloway JW, et al. Identification of ATPAF1 as a novel candidate gene for asthma in children. J Allergy Clin Immunol. 2011;128(4):753,760.e11.
152. Sleiman PM, Flory J, Imielinski M, Bradfield JP, Annaiah K, Willis-Owen SA, et al. Variants of DENND1B associated with asthma in children. N Engl J Med. 2010;362(1):36-44.
153. Anantharaman R, Andiappan AK, Nilkanth PP, Suri BK, Wang de Y, Chew FT. Genome-wide association study identifies PERLD1 as asthma candidate gene. BMC Med Genet. 2011;12:170.
154. Hancock DB, Romieu I, Shi M, Sienra-Monge JJ, Wu H, Chiu GY, et al. Genome-wide association study implicates chromosome 9q21.31 as a susceptibility locus for asthma in mexican children. PLoS Genet. 2009;5(8):e1000623.

155. Liew FY, Pitman NI, McInnes IB. Disease-associated functions of IL-33: The new kid in the IL-1 family. Nat Rev Immunol. 2010;10(2):103-10.
156. Cantero-Recasens G, Fandos C, Rubio-Moscardo F, Valverde MA, Vicente R. The asthma-associated ORMDL3 gene product regulates endoplasmic reticulum-mediated calcium signaling and cellular stress. Hum Mol Genet. 2010;19(1):111-21.
157. Koppelman GH. Gene by environment interaction in asthma. Curr Allergy Asthma Rep. 2006;6(2):103-11.
158. Vercelli D. Gene-environment interactions in asthma and allergy: The end of the beginning? Curr Opin Allergy Clin Immunol. 2010;10(2):145-8.
159. Bottema RW, Kerkhof M, Reijmerink NE, Thijs C, Smit HA, van Schayck CP, et al. Gene-gene interaction in regulatory T-cell function in atopy and asthma development in childhood. J Allergy Clin Immunol. 2010;126(2):338,46, 346.e1-10.
160. Smit LA, Heederik D, Doekes G, Koppelman GH, Bottema RW, Postma DS, et al. Endotoxin exposure, CD14 and wheeze among farmers: A gene--environment interaction. Occup Environ Med. 2011;68(11):826-31.
161. Ege MJ, Strachan DP, Cookson WO, Moffatt MF, Gut I, Lathrop M, et al. Gene-environment interaction for childhood asthma and exposure to farming in central europe. J Allergy Clin Immunol. 2011;127(1):138,44, 144.e1-4.
162. Kerkhof M, Postma DS, Brunekreef B, Reijmerink NE, Wijga AH, de Jongste JC, et al. Toll-like receptor 2 and 4 genes influence susceptibility to adverse effects of traffic-related air pollution on childhood asthma. Thorax. 2010;65(8):690-7.
163. Reijmerink NE, Kerkhof M, Bottema RW, Gerritsen J, Stelma FF, Thijs C, et al. Toll-like receptors and microbial exposure: Gene-gene and gene-environment interaction in the development of atopy. Eur Respir J. 2011;38(4):833-40.
164. Custovic A, Rothers J, Stern D, Simpson A, Woodcock A, Wright AL, et al. Effect of day care attendance on sensitization and atopic wheezing differs by toll-like receptor 2 genotype in 2 population-based birth cohort studies. J Allergy Clin Immunol. 2011;127(2):390,397.e1-9.
165. Sano K, Tanihara H, Heimark RL, Obata S, Davidson M, St John T, et al. Protocadherins: A large family of cadherin-related molecules in central nervous system. EMBO J. 1993;12(6):2249-56.
166. Hulpiau P, van Roy F. Molecular evolution of the cadherin superfamily. Int J Biochem Cell Biol. 2009;41(2):349-69.
167. Kim SY, Yasuda S, Tanaka H, Yamagata K, Kim H. Non-clustered protocadherin. Cell Adh Migr. 2011; 5(2):97-105.
168. Redies C, Vanhalst K, Roy F. Delta-protocadherins: Unique structures and functions. Cell Mol Life Sci. 2005;62(23):2840-52.
169. Huang YT, Heist RS, Chirieac LR, Lin X, Skaug V, Zienolddiny S, et al. Genome-wide analysis of survival in early-stage non-small-cell lung cancer. J Clin Oncol. 2009;27(16):2660-7.
170. Carrasquillo MM, Zou F, Pankratz VS, Wilcox SL, Ma L, Walker LP, et al. Genetic variation in PCDH11X is associated with susceptibility to late-onset alzheimer's disease. Nat Genet. 2009;41(2):192-8.
171. Wang C, Yu G, Liu J, Wang J, Zhang Y, Zhang X, et al. Downregulation of PCDH9 predicts prognosis for patients with glioma. J Clin Neurosci. 2012;19(4):541-5.
172. Kuroda H, Inui M, Sugimoto K, Hayata T, Asashima M. Axial protocadherin is a mediator of prenotochord cell sorting in xenopus. Dev Biol. 2002;244(2):267-77.
173. Patel SD, Chen CP, Bahna F, Honig B, Shapiro L. Cadherin-mediated cell-cell adhesion: Sticking together as a family. Curr Opin Struct Biol. 2003;13(6):690-8.
174. Morishita H, Umitsu M, Murata Y, Shibata N, Udaka K, Higuchi Y, et al. Structure of the cadherin-related neuronal receptor/protocadherin-alpha first extracellular cadherin domain reveals diversity across cadherin families. J Biol Chem. 2006;281(44):33650-63.
175. Elledge HM, Kazmierczak P, Clark P, Joseph JS, Kolatkar A, Kuhn P, et al. Structure of the N terminus of cadherin 23 reveals a new adhesion mechanism for a subset of cadherin superfamily members. Proc Natl Acad Sci U S A. 2010;107(23):10708-12.
176. Vanhalst K, Kools P, Staes K, van Roy F, Redies C. Delta-protocadherins: A gene family expressed differentially in the mouse brain. Cell Mol Life Sci. 2005;62(11):1247-59.
177. Fitsialos G, Chassot AA, Turchi L, Dayem MA, LeBrigand K, Moreilhon C, et al. Transcriptional signature of epidermal keratinocytes subjected to in vitro scratch wounding reveals selective roles for ERK1/2, p38, and phosphatidylinositol 3-kinase signaling pathways. J Biol Chem. 2007;282(20):15090-102.
178. Colland F, Jacq X, Trouplin V, Mougin C, Groizeleau C, Hamburger A, et al. Functional proteomics mapping of a human signaling pathway. Genome Res. 2004;14(7):1324-32.

Chapter 2

Identification of *PCDH1* as a novel susceptibility gene for bronchial hyperresponsiveness

Gerard H. Koppelman
Deborah A. Meyers
Timothy D. Howard
S. Lilly Zheng
Greg A. Hawkins
Elizabeth J. Ampleford
Jianfeng Xu
Henk Koning
Marcel Bruinenberg
Ilja M. Nolte
Cleo C. van Diemen
H. Marike Boezen
Wim Timens
Paul A. Whittaker
O. Colin Stine
Sheila J. Barton
John W. Holloway
Stephen T. Holgate
Penelope E. Graves
Fernando D. Martinez
Antoon J.M. van Oosterhout
Eugene R. Bleecker
Dirkje S. Postma

Am J Respir Crit Care Med. 2009. Vol 180(10): p929-935

ABSTRACT

Rationale. Asthma is a chronic inflammatory airway disease that affects over 300 million individuals worldwide. Asthma is caused by interaction of genetic and environmental factors. Bronchial hyperresponsiveness (BHR) is a hallmark of asthma and results from increased sensitivity of the airways to physical or chemical stimulants. BHR and asthma are linked to chromosome 5q31-q33.

Objective. To identify a gene for BHR on chromosome 5q31-33

Methods. In 200 Dutch asthma families, linkage analysis and fine mapping was performed, and the *Protocadherin 1 gene* (*PCDH1*) was identified. *PCDH1* was resequenced in 96 subjects from ethnically diverse populations to identify novel sequence variants. Subsequent replication studies were undertaken in seven populations from the Netherlands, UK and USA, including two general population samples, two family and three case-control samples. PCDH1 mRNA and protein expression was investigated using PCR, Western blotting and immunohistochemistry.

Main results. In seven out of eight populations (n = 6,168) from the Netherlands, UK, and USA, *PCHD1* gene variants were significantly associated with BHR (p values: 0.005 - 0.05) This association was present in both asthma families and general populations. PCDH1 mRNA and protein was expressed in airway epithelial cells, and in macrophages.

Conclusion. PCDH1 is a novel gene for BHR in adults and children. The identification of *PCDH1* as a BHR susceptibility gene may suggest that a structural defect in the integrity of the airway epithelium, the first line of defence against inhaled substances, contributes to the development of BHR.

INTRODUCTION

Asthma is a chronic inflammatory airway disease that affects over 300 million individuals worldwide (1). It is characterized by respiratory symptoms, variable airway obstruction, and bronchial hyperresponsiveness (BHR) and caused by multiple genetic and environmental factors that may interact (2). BHR is a hallmark of asthma and is due to increased sensitivity of the airways to physical or chemical stimulants (cold air and cigarette smoke) and to pharmacological agents such as methacholine and histamine. BHR has a considerable genetic component (3) and constitutes a risk factor for asthma development, even in subjects without respiratory symptoms (4). Furthermore, BHR is increased in children who are exposed to environmental tobacco smoke (ETS) in utero and in the first years of life (5).

Our initial report of linkage of bronchial hyperresponsiveness (BHR) to chromosome 5q31-q33 in Dutch asthma families (6) has been confirmed in six other populations for asthma associated phenotypes (7-12). This finding has been extended by showing that chromosome 5q31-q33 interacts with ETS exposure in utero and/or early childhood in the development of BHR and asthma (13, 14). This region of chromosome 5q31-q33 contains a large number of candidate genes for allergy and asthma, such as *Interleukin-13*, *Interleukin-9*, *Interleukin-4*, *CD14*, *IRF-1*, *GM-CSF*, *TIM-1* and the *beta-2- adrenoceptor* (15). These genes have been reported to be associated with asthma associated phenotypes, which strongly suggests that chromosome 5q31-q33 contains multiple asthma susceptibility genes that each contribute to the observed asthma linkage.

In this article, we report the identification of protocadherin-1 (*PCDH1)* as a novel gene for BHR on chromosome 5q31-33. We used positional cloning to identify this gene in the Dutch population, subsequently identified novel sequence variants, performed genetic replication studies in seven independent populations in the Netherlands, the USA and the United Kingdom and show that PCDH1 mRNA and protein expression is present in airway epithelium, the first line of defence against inhaled allergens and toxic substances known to contribute to asthma development.

MATERIAL AND METHODS

Study populations

Recruitment and clinical characterization of all study samples are described in detail in the Supplementary Data. The primary population consists of two hundred families (1259 individuals) ascertained through probands with asthma who were initially studied between 1962 and 1975 at Beatrixoord Hospital, Haren, The Netherlands (Supplementary Table E1) (16). The Dutch replication samples include an asthma trio population of 407 trios (17), and the longitudinal Vlagtwedde/Vlaardingen cohort study, that includes subjects from the general population that have been tested for bronchial hyperresponsiveness at one or more time points during follow up (n=418) (Supplementary Table E2) (18).

Replication samples from the United States include the prospective Children's Respiratory Study *(Tucson, AZ, USA),* in which children were tested for BHR to methacholine at age 11 and 16 (n=329) (table E2) (19). Furthermore, three US case control populations were recruited from three ethnically diverse US populations (African American (n=522), Hispanic (n=246), and Caucasian (n=665)) as part of the Collaborative Study on the Genetics of Asthma (20, 21). BHR measurements to methacholine or reversibility to albuterol were performed in the cases (Supplementary Table E2) (21).

The replication sample from the United Kingdom is an asthma family study from Southampton, UK, which includes 341 affected sib pair families with asthma (n=1508) (22). Bronchial responsiveness was measured in subjects with a baseline FEV_1 of >70% predicted using inhaled methacholine (Supplementary Table E2) (22). Local medical ethics committees approved these studies, and all participants provided written informed (parental) consent.

Genotyping and sequence analysis

Genotyping methods and marker selection are described in the Supplementary Data. Briefly, 22 microsatellite markers were identified on chromosome 5q31-33 and genotyped in the Dutch family study (14). Subsequently, 103 SNPs were selected for fine mapping and genotyped in the Dutch family study (Supplementary Table E3). The *PCDH1* gene (2 kb of the putative promoter region, all exons, and the complete 3' untranslated region (UTR) region) was resequenced in 96 subjects as described previously (23).

Statistical analysis

Linkage analysis of the microsatellite fine screen markers on chromosome 5q31-33 was performed using GENEHUNTER-PLUS, using the same model as described previously (14).

Association analysis. Dutch families, trios and UK families were analyzed with FBAT using an additive model, using the –e option in case of linkage (24). Case-control analysis was performed using Chisquare tests and ANOVA, if appropriate. In the Vlagtwedde/ Vlaardingen cohort study, the association between *PCDH1* SNPs and the occurrence of becoming BHR positive over time was investigated longitudinally using a Cox regression model (SPSS 14.0), with adjustment for smoking, age at investigation and FEV_1 at baseline. All statistical tests were performed two sided.

PCDH1 expression

PCDH1 mRNA expression was investigated using PCR in mRNA obtained from an epithelial cell line 16HBE, and human cells including a.o. blood cells, brain, lung fibroblasts as well as cultured airway epithelial cells of asthma patients. PCDH1 protein expression was investigated using two different antibodies (a polyclonal and a monoclonal antibody) using Western Blotting and immunohistochemisty as described in the Supplementary Data.

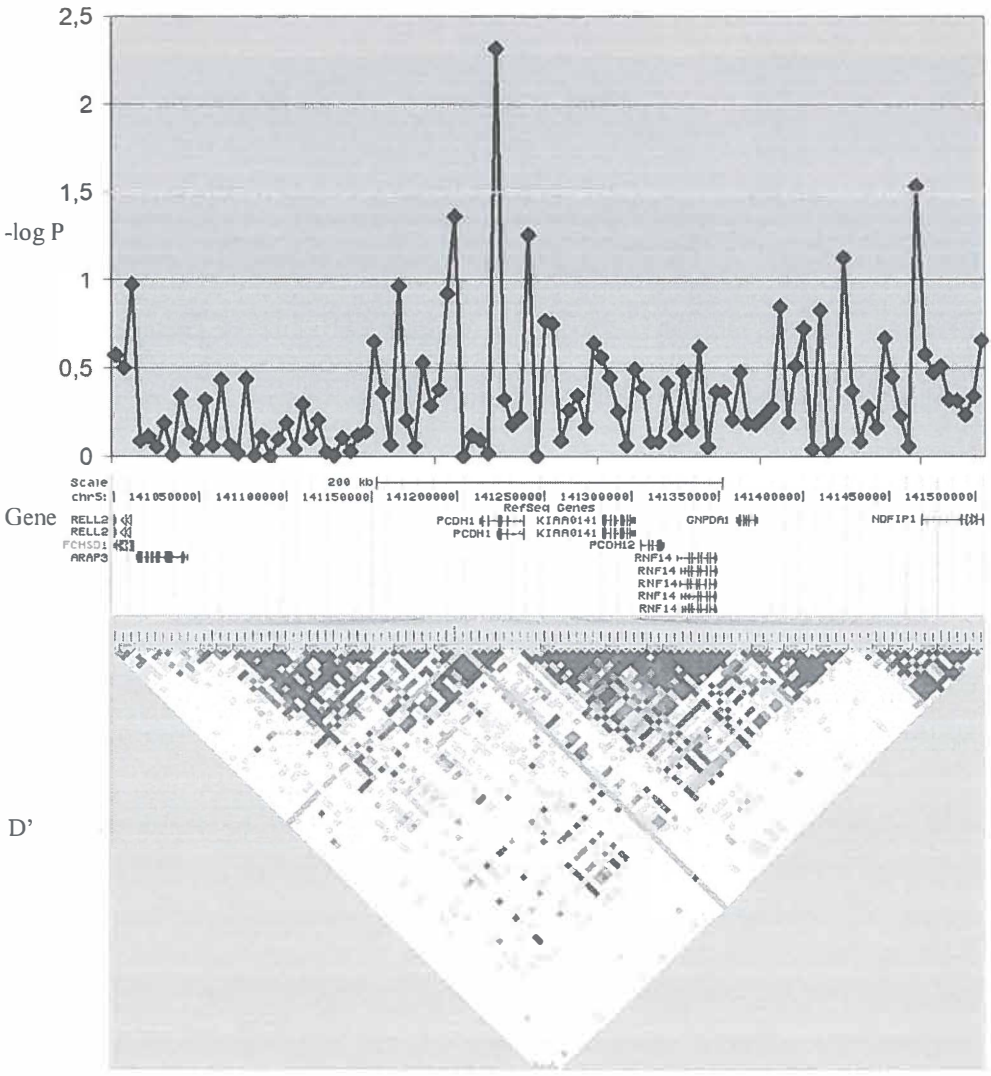
RESULTS

Linkage analysis using a dense map of 29 microsatellite markers in the linked region on chromosome 5q31-q33 in 200 Dutch families (n=1,259) revealed significant LOD scores in two regions, i.e. HLOD was 3.88 at marker 5q31-33_36 and 3.84 in a region flanked by markers D5S2117 and *IL9*. In 95 ETS-exposed families, a peak HLOD score of 4.36 was flanked by markers 5q31-33_56 and *FGFα* (Supplementary Figure E1).

Using the approach previously used to identify *DPP10* and *PHF11*, two positionally cloned genes for asthma (25, 26), allelic association of microsatellite markers and BHR was tested. One allele of marker 5q31-33_40 was significantly associated with BHR using probands and spouses in a case-control design (p= 0.0029). This marker is located next to the peak LOD score in the ETS-exposed families at 142.13 centiMorgan (cM); its physical position is at 136,413,897-136,414,296 on the reference sequence. The 250 kb region surrounding this marker was investigated, based on the extent of linkage disequilibrium between markers on 5q in the Dutch population. 103 Haplotype tagging and functional single nucleotide polymorphisms (SNPs) were genotyped and family based association tests identified significant associations of SNPs in *protocadherin-1* (*PCDH1*) with BHR, and specifically

with rs3797054, which encodes a synonymous SNP (Ala750Ala) in *PCDH1* (p=0.005) (Figure 1).

Figure 1: Linkage disequilibrium (D') and association of SNPs at chromosome 5q31-q33 and BHR in the Dutch family study



Resequencing of *PCDH1* in 96 subjects of ethnically diverse populations revealed 22 SNPs and two insertion deletion polymorphisms (Supplementary Table E4).

Potential functional SNPs significantly associated with BHR were genotyped in seven replication populations from the Netherlands (adults), the United States (adults and children) and the United Kingdom (children), displaying significant associations in two Dutch, one UK and three US populations (Table 1).

Table 1: Association of PCDH1 gene variants with bronchial hyperresponsiveness in 8 populations (n=6,168)

SNP	Rs number	Risk allele (All populations)	Allele frequency in Dutch families	Netherlands 200 Asthma families	Netherlands 200 Asthma parents (case-control)	Netherlands 407 asthma trios	Netherlands General population. adults	Tucson. AZ General population Children Aged 11 and 16 y		Southampton. UK 341 families with asthma. children	US-White (W): Hispanic (H) and Afro-American (A). Case-control
N				1259	401	1221^	418	318*	329*	1508	W: 338 cases. 327controls H 116 cases. 130 controls A: 222 cases. 300 controls
Analysis				FBAT	Chi-square	FBAT	Cox regression	ANOVA	ANOVA	FBAT	Chi square. ANOVA
-1191	RS13355929	A	0.03	0.45	0.31	**0.09**	-	-	-	-	Ns
Ala514Thr	RS3822357	G	0.92	0.47	0.11	0.72	0.58	0.07	**0.005** '	**0.009**	Ns
Ala750Ala	RS3797054	T	0.67	**0.005**	**0.02**	0.49	**0.05**	**0.04** #	0.28	0.19	Ns
IVS3+164	RS14359	C	-	-	0.45	0.93	0.09	0.37	1.0	0.18	Ns
IVS3-116	-	Del TTC	0.08	**0.04**	0.25	**0.05**	0.95	§	§	0.58	**0.02 (C, BHR)**

Definition of abbreviations: ANOVA = analysis of variance; BHR = bronchial hyperresponsiveness; Del = Deletion; FBAT = Family Based Association Tests; Rs = Ref SNP accession ID; SNP = single nucleotide polymorphism.
Significant cases appear in bold. US case control populations:BHR was analyzed in cases only, as BHR was not measured in the controls. Due to low numbers of Hispanic cases, power was low for BHR analysis and therefore asthma was analyzed (p = 0.02 for IVS3_116). In all polulations, the same risk allele was tested for association.
 # dominant model (p value for ANOVA 2 d.f. = 0.13)
^ Number represents total number of subjects of these trios, including 407 asthma probands.
** Only children with BHR measurements were included in these analyses.*
§ Data not shown due to differences in allele frequency of Hispanic and Caucasian populations.

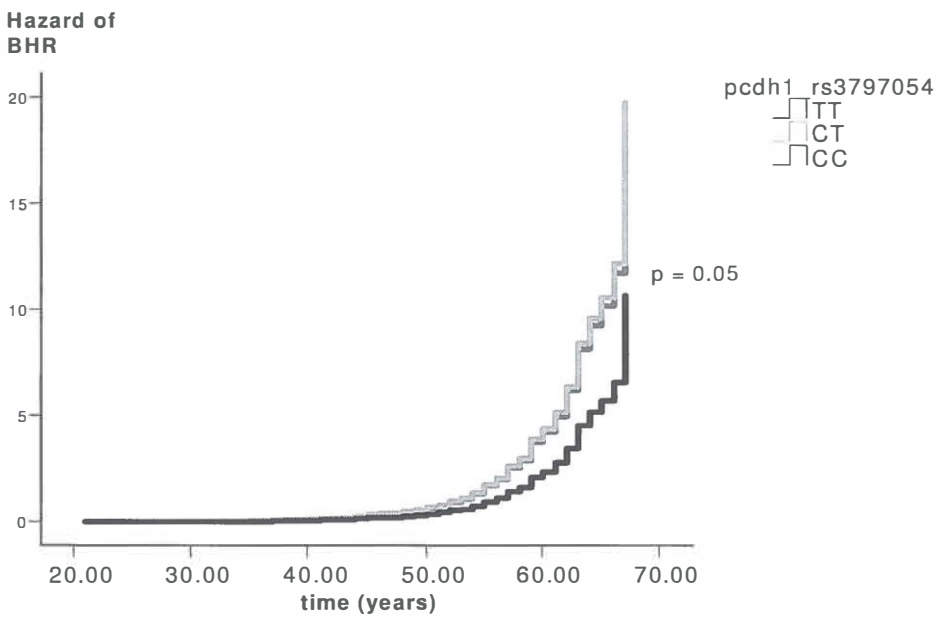
First, the association found between rs3797054 (T-allele) and BHR was consistently observed in the parents of the Dutch families (using the probands with asthma as cases and unaffected spouses in a case-control design) and in the children of the Dutch families (using FBAT). The association of rs3797054 with BHR was replicated in participants of the population-based Children's Respiratory Study in Tucson, Arizona, USA, who were investigated for BHR at age 11 and age 16 (19). Moreover, rs3797054 was associated with the development of BHR in adults from a longitudinal adult population-based study in the Netherlands (Figure 2) (18). Second, a three base pair insertion/deletion polymorphism (IVS3 -116) in the intronic region of exon 3 was associated with BHR in two Dutch populations and with asthma and BHR in two US case-control populations. Third, the major allele of rs3822357, which encodes Ala514Thr localised in the 5th cadherin repeat, was associated with BHR in the US Children

Respiratory Study (19), and with BHR in a UK population of 341 families ascertained through affected siblings with asthma (22). Interestingly, *PCDH1* Ala514Thr was associated with BHR when ETS exposure in utero and the first years of life was taken into account in the latter population (Table 1). However, there was not strong evidence for gene by smoking interaction in the Dutch or the US Tucson populations.

In addition to BHR, we investigated *PCDH1* SNPs in asthma defined by an algorithm (14) and observed significant association of rs3797054 and asthma (p = 0.003) in the 200 Dutch families. Haplotype analysis did not further improve these results.

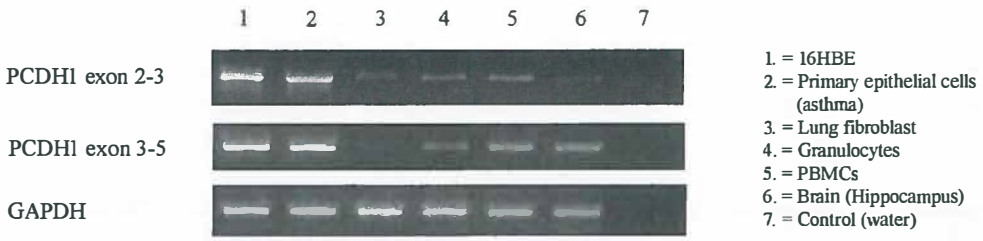
Figure 2: Longitudinal analysis of PCDH1 Ala750Ala in the general Dutch population.

Hazard function of developing BHR in the general Dutch population of Vlagtwedde-Vlaardingen over time. PCDH1 CT or TT carriers are at increased risk of becoming hyperresponsive over time compared to PCDH1 Ala750Ala CC homozygotes (p=0.05)



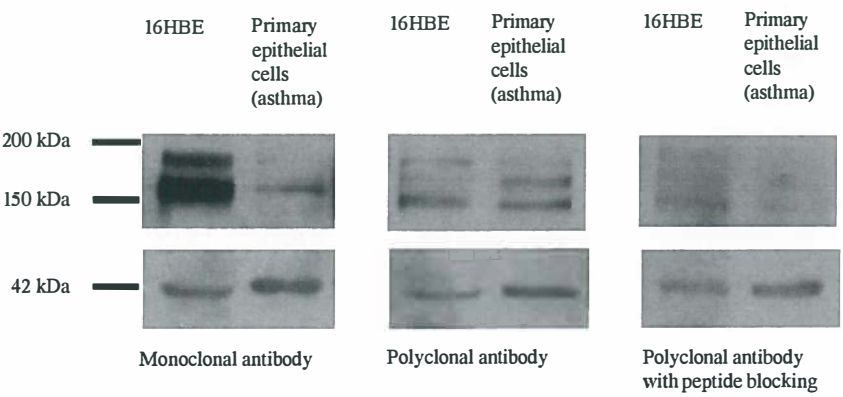
By PCR, we identified high *PCDH1* mRNA expression in human brain, airway epithelial cell lines (16HBE) and in primary epithelial cell cultures of asthma patients. Weak mRNA expression was observed in airway fibroblasts, peripheral blood mononuclear cells and granulocytes (Figure 3).

Figure 3: mRNA expression of PCDH1 in various tissues and cells.



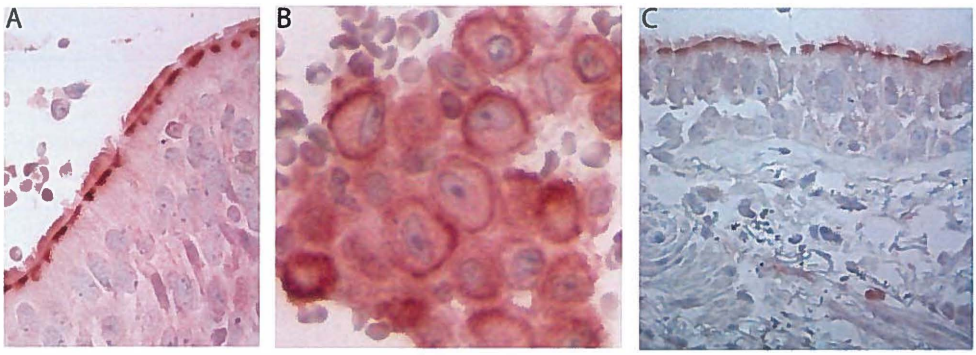
Western blot analysis showed consistent and specific PCDH1 protein expression in bronchial epithelial cell line 16HBE and primary epithelial cell cultures of asthma patients using different polyclonal and monoclonal antibodies. Proteins of 150-160 kD were identified with a protein weight consistent with previous observations (Figure 4) (27).

Figure 4: Western Blot of epithelial cell line 16 HBE, and primary epithelial cell culture of an asthma patient using a monoclonal and a different polyclonal antibody.
Upper lane PCDH1, lower lane β-actin. One experiment of n=4 is shown. Protein expression was identified using two different antibodies, a monoclonal (Abnova, Taiwan) (left panel) and a polyclonal antibody (Eurogentec, Liege, Belgium) (middle panel). The signal of the polyclonal antibody was blocked by preincubation with peptide (right panel).



Immunohistochemistry using the PCDH1 polyclonal antibody showed a specific expression pattern of PCDH1 protein in the apical part of differentiated airway epithelial cells lining the airway lumen and in the membrane of macrophages in lung resection specimens of a patient with bronchitis as well as airway wall biopsies in asthma patients (Figure 5). PCDH1 expression was also observed between epithelial cells at the apical side of the epithelium.

Figure 5: Protein expression of PCDH1 in airway epithelial cells and macrophages.
Protein expression of PCDH1 in airway epithelial cells and macrophages on lung (panel a and b) and airway wall biopsy of asthma patient; panel c). Expression is observed at the apical part of airway epithelial cells (panel a and c) and in macrophages (panel b).



DISCUSSION

This study identified *PCDH1* as a novel gene for BHR in children and adults. Interestingly, we have provided evidence that *PCDH1*, particularly rs3797054, is important in the development of BHR in an asthma family sample. In addition, *PCDH1* is associated with BHR in two population based samples from the Netherlands and the US, irrespective of the presence of asthma. Moreover, we present strict replication of a second gene variant in *PCDH1*, a three base pair insertion-deletion, in three additional study samples ascertained for asthma. Finally, loose replication (28) was observed in the Tucson cohort and a UK family study for a coding SNP not associated with BHR in the primary Dutch population. Of importance for the relevance of the *PCDH1* gene for BHR and asthma, *PCDH1* mRNA and protein expression was shown in macrophages and airway epithelial cells in asthma and controls.

In order to interpret these findings, several strengths and limitations need to be considered. First, to our knowledge this is the first gene that is identified by positional cloning for an intermediate phenotype of asthma, bronchial hyperresponsiveness. *PCDH1* gene variants are associated with BHR in asthma families as well as two general populations, not ascertained for asthma. Previous epidemiological data has shown that BHR is a risk factor for asthma, even in subjects without respiratory symptoms (4). Based on these data, we

hypothesize that mechanisms related to the function of *PCDH1* contribute to susceptibility to BHR and subsequent asthma development. This may implicate that PCDH1 dysfunction plays an early role in asthma pathogenesis.

Second, in all study populations, a direct agonist of BHR was used (either histamine or methacholine). Our findings suggest that PCDH1 function is not specific for BHR induced by either methacholine or histamine. Previous work has indicated that BHR to histamine and methacholine is highly correlated (29). We suggest that further work should be performed on the role of *PCDH1* in indirect measures of BHR, such as exercise or adenosine-mono-phosphate.

Third, we used the positional cloning approach that has been previously used to identify *DPP10* and *PHD11* (25, 26). This approach is based on identification of a genetic association with a microsatellite marker used for linkage analyses, to subsequently fine map the region that is in possible LD with this marker and the gene of interest. Our fine mapping results indicate that we have adequately screened the region for SNPs in other genes that may be in LD with *PCDH1*, given the extent of LD of about 100 kB in this region in the Dutch population (Figure 1).

Fourth, we identified *PCDH1* in a primary family population, in which linkage to BHR was reported (6, 14). We did not correct for multiple testing, but rather performed extensive replication studies of significant and/or functional SNPs in 7 independent populations. We interpret the consistent signal in parents and offspring in the Dutch families as internal validation of this genetic association in the Dutch families. Moreover, the strict replication of two *PCDH1* gene variants with the same risk allele being associated with BHR in the same direction in 4 independent populations provides strong support for a role of *PCDH1* in BHR (28). Specifically, we found strict replication with regard to phenotype and genotype for Ala750Ala and IVS3_116. Loose replication with regard to genotype was observed for Ala514Thr. The reasons for this allelic heterogeneity are yet to be determined, but include the presence of multiple functional SNPs, other SNPs that are in linkage disequilibrium with the associated SNPs or gene by environment interaction. Since gene variants on 5q31-33 have been shown to interact with ETS exposure in utero/early life, we investigated the association of *PCDH1* with BHR in ETS exposed and non-exposed children in four populations. These gene-environmental interaction analyses (Supplementary Table E5) in the study cohorts revealed evidence of gene environment interaction for *PCDH1* in the UK family cohort only, but not in the other populations. However, the power to detect such an interaction was low.

We suggest characterizing the functional role of these gene variants in *PCDH1*. Ala750Ala and IVS3_116 are localised in the 3'UTR and intronic region of exon 3 and may affect mRNA stability or splicing; whereas Ala514Thr is localised in the 5th cadherin repeat of the extracellular domain and may affect cell-cell adhesion.

The *Protocadherin-1* gene (*PCDH1*, *PC42*) has 5 exons and encodes multiple mRNA isoforms through alternative splicing (Supplementary Table E4). There are two annotated isoforms: a 3 and 5 exon isoform. The three exon isoform lacks the major part of the cytoplasmic domain, which encodes conserved signalling sequences CM1, CM2 and CM3 (22). Protocadherins are thought to play an important role in homotypic cell adhesion and organ development, in particular within the neural system (30). Overexpression of PCDH1 induces calcium dependent cell-cell adhesion and membrane expression of PCDH1 in a mouse fibroblast L-cell assay (27). Protocadherin-1 belongs to the δ1-protocadherin family of transmembrane proteins, together with a.o. PCDH7. δ1-protocadherins are characterized by 7 cadherin repeats in the extracellular region, and three conserved regions designated CM1, -2 and -3 in the intracellular domain (31). The conserved region CM3 interacts with protein phosphatase 1 α (PP1α) in PCDH7 (31). Interestingly, PP1α plays an important role in lung development, as inhibition of PP1α in a mouse model led to impaired lung development and branching morphogenesis (32). Finally, in a skin keratinocyte wounding model, *PCDH1* mRNA was significantly upregulated 24 hours after wounding, compatible with a role of PCDH1 in epithelial repair (33).

The expression pattern of PCDH1 is consistent with expression in the apical adhesion complex of airway epithelial cells. We therefore hypothesize that PCDH1 plays a role in epithelial integrity of the airways and that loss of function of PCDH1 is associated with increased BHR which may lead to symptomatic asthma (34). It is tempting to speculate that PCDH1 dysfunction may provide a functional explanation for the observed epithelial vulnerability and increased epithelial shedding in asthma (35). Further investigations will address the functional relevance of genetic variations in *PCDH1* in epithelial cell adhesion and its interaction with environmental tobacco smoke exposure. Moreover, these finding may be relevant for other diseases in which the integrity of the epithelium is a potential pathogenetic mechanism, such as atopic dermatitis (36) and celiac disease (37). We therefore suggest performing genetic studies of *PCDH1* in these diseases.

In conclusion, this is the first report of a gene specifically identified for BHR, an important hallmark of asthma. Further investigations in PCDH1 function may provide novel insight into its role in the integrity of the airway epithelium in BHR and asthma development.

ACKNOWLEDGEMENTS

We would like to thank all participants of all studies, and the lung function department of Beatrixoord hospital who assisted in the clinical testing. In addition, we would like to thank the collaborating Frisian pulmonologists for their help in patient recruitment. Furthermore, we are thankful to G. van der Steege, G.J. te Meerman, D. de Jong, M.N. Hylkema, A. Blacquire, M. Geerlings, M. Nawijn, M.T. Hanley, A. Bench and J. Cakebread for advice and technical assistance.

REFERENCES

1. Masoli, M, Fabian, D., Holt, S., and Global Initiative for Asthma (GINA) program. Global burden of asthma: executive summary of the GINA Dissemination Committee report. Allergy 59[5], 469-478. 2004.
2. Koppelman, G. H. 2006. Gene by environment interaction in asthma. Curr.Allergy Asthma Rep. 6:103-111.
3. Hopp, R. J., A. K. Bewtra, G. D. Watt, N. M. Nair, and R. G. Townley. 1984. Genetic analysis of allergic disease in twins. J.Allergy Clin.Immunol. 73:265-270.
4. Rasmussen, F., D. R. Taylor, E. M. Flannery, J. O. Cowan, J. M. Greene, G. P. Herbison, and M. R. Sears. 2002. Outcome in adulthood of asymptomatic airway hyperresponsiveness in childhood: a longitudinal population study. Pediatr.Pulmonol. 34:164-171.
5. Cook, D. G. and D. P. Strachan. 1998. Parental smoking, bronchial reactivity and peak flow variability in children. Thorax 53:295-301.
6. Postma, D. S., E. R. Bleecker, P. J. Amelung, K. J. Holroyd, J. Xu, C. I. Panhuysen, D. A. Meyers, and R. C. Levitt. 1995. Genetic susceptibility to asthma--bronchial hyperresponsiveness coinherited with a major gene for atopy. N.Engl.J.Med. 333:894-900.
7. Noguchi, E., M. Shibasaki, T. Arinami, K. Takeda, T. Maki, T. Miyamoto, T. Kawashima, K. Kobayashi, and H. Hamaguchi. 1997. Evidence for linkage between asthma/atopy in childhood and chromosome 5q31-q33 in a Japanese population. Am.J.Respir.Crit Care Med. 156:1390-1393.
8. Yokouchi, Y., Y. Nukaga, M. Shibasaki, E. Noguchi, K. Kimura, S. Ito, M. Nishihara, K. Yamakawa-Kobayashi, K. Takeda, N. Imoto, K. Ichikawa, A. Matsui, H. Hamaguchi, and T. Arinami. 2000. Significant evidence for linkage of mite-sensitive childhood asthma to chromosome 5q31-q33 near the interleukin 12 B locus by a genome-wide search in Japanese families. Genomics 66:152-160.
9. Walley, A. J., S. Wiltshire, C. M. Ellis, and W. O. Cookson. 2001. Linkage and allelic association of chromosome 5 cytokine cluster genetic markers with atopy and asthma associated traits. Genomics 72:15-20.
10. Shek, L. P., A. H. Tay, F. T. Chew, D. L. Goh, and B. W. Lee. 2001. Genetic susceptibility to asthma and atopy among Chinese in Singapore--linkage to markers on chromosome 5q31-33. Allergy 56:749-753.
11. Holloway, J. W., C. Lonjou, B. Beghe, Q. Peng, T. R. Gaunt, I. Gomes, I. P. Hall, J. C. Dewar, J. Wilkinson, N. S. Thomas, S. T. Holgate, and N. E. Morton. 2001. Linkage analysis of the 5q31-33 candidate region for asthma in 240 UK families. Genes Immun. 2:20-24.
12. Haagerup, A., T. Bjerke, P. O. Schiotz, H. G. Binderup, R. Dahl, and T. A. Kruse. 2002. Asthma and atopy - a total genome scan for susceptibility genes. Allergy 57:680-686.
13. Colilla, S., D. Nicolae, A. Pluzhnikov, M. N. Blumenthal, T. H. Beaty, E. R. Bleecker, E. M. Lange, S. S. Rich, D. A. Meyers, C. Ober, and N. J. Cox. 2003. Evidence for gene-environment interactions in a linkage study of asthma and smoking exposure. J.Allergy Clin.Immunol. 111:840-846.

14. Meyers, D. A., D. S. Postma, O. C. Stine, G. H. Koppelman, E. J. Ampleford, H. Jongepier, T. D. Howard, and E. R. Bleecker. 2005. Genome screen for asthma and bronchial hyperresponsiveness: interactions with passive smoke exposure. J.Allergy Clin.Immunol. 115:1169-1175.
15. Ober, C. and S. Hoffjan. 2006. Asthma genetics 2006: the long and winding road to gene discovery. Genes Immun. 7:95-100.
16. Panhuysen, C. I., E. R. Bleecker, G. H. Koeter, D. A. Meyers, and D. S. Postma. 1998. Characterization of obstructive airway disease in family members of probands with asthma. An algorithm for the diagnosis of asthma. Am.J.Respir.Crit Care Med. 157:1734-1742.
17. Jongepier, H., G. H. Koppelman, I. M. Nolte, M. Bruinenberg, E. R. Bleecker, D. A. Meyers, G. J. te Meerman, and D. S. Postma. 2005. Polymorphisms in SPINK5 are not associated with asthma in a Dutch population. J.Allergy Clin.Immunol. 115:486-492.
18. van Diemen, C. C., D. S. Postma, J. M. Vonk, M. Bruinenberg, J. P. Schouten, and H. M. Boezen. 2005. A disintegrin and metalloprotease 33 polymorphisms and lung function decline in the general population. Am.J.Respir.Crit Care Med. 172:329-333.
19. Stein, R. T., C. J. Holberg, W. J. Morgan, A. L. Wright, E. Lombardi, L. Taussig, and F. D. Martinez. 1997. Peak flow variability, methacholine responsiveness and atopy as markers for detecting different wheezing phenotypes in childhood. Thorax 52:946-952.
20. Basehore, M. J., T. D. Howard, L. A. Lange, W. C. Moore, G. A. Hawkins, P. L. Marshik, M. S. Harkins, D. A. Meyers, and E. R. Bleecker. 2004. A comprehensive evaluation of IL4 variants in ethnically diverse populations: association of total serum IgE levels and asthma in white subjects. J.Allergy Clin.Immunol. 114:80-87.
21. Xu, J., D. A. Meyers, C. Ober, M. N. Blumenthal, B. Mellen, K. C. Barnes, R. A. King, L. A. Lester, T. D. Howard, J. Solway, C. D. Langefeld, T. H. Beaty, S. S. Rich, E. R. Bleecker, and N. J. Cox. 2001. Genomewide screen and identification of gene-gene interactions for asthma-susceptibility loci in three U.S. populations: collaborative study on the genetics of asthma. Am.J.Hum.Genet. 68:1437-1446.
22. Van Eerdewegh, P., R. D. Little, J. Dupuis, R. G. Del Mastro, K. Falls, J. Simon, D. Torrey, S. Pandit, J. McKenny, K. Braunschweiger, A. Walsh, Z. Liu, B. Hayward, C. Folz, S. P. Manning, A. Bawa, L. Saracino, M. Thackston, Y. Benchekroun, N. Capparell, M. Wang, R. Adair, Y. Feng, J. Dubois, M. G. FitzGerald, H. Huang, R. Gibson, K. M. Allen, A. Pedan, M. R. Danzig, S. P. Umland, R. W. Egan, F. M. Cuss, S. Rorke, J. B. Clough, J. W. Holloway, S. T. Holgate, and T. P. Keith. 2002. Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness. Nature 418:426-430.
23. Hawkins, G. A., P. J. Amelung, R. S. Smith, H. Jongepier, T. D. Howard, G. H. Koppelman, D. A. Meyers, E. R. Bleecker, and D. S. Postma. 2004. Identification of polymorphisms in the human glucocorticoid receptor gene (NR3C1) in a multi-racial asthma case and control screening panel. DNA Seq. 15:167-173.
24. Horvath, S., X. Xu, and N. M. Laird. 2001. The family based association test method: strategies for studying general genotype--phenotype associations. Eur.J.Hum.Genet. 9:301-306.
25. Zhang, Y., N. I. Leaves, G. G. Anderson, C. P. Ponting, J. Broxholme, R. Holt, P. Edser, S. Bhattacharyya, A. Dunham, I. M. Adcock, L. Pulleyn, P. J. Barnes, J. I. Harper, G. Abecasis, L. Cardon, M. White, J. Burton, L. Matthews, R. Mott, M. Ross, R. Cox, M. F. Moffatt, and W. O. Cookson. 2003. Positional cloning of a quantitative trait locus on chromosome 13q14 that influences immunoglobulin E levels and asthma. Nat.Genet. 34:181-186.
26. Allen, M., A. Heinzmann, E. Noguchi, G. Abecasis, J. Broxholme, C. P. Ponting, S. Bhattacharyya, J. Tinsley, Y. Zhang, R. Holt, E. Y. Jones, N. Lench, A. Carey, H. Jones, N. J. Dickens, C. Dimon, R. Nicholls, C. Baker, L. Xue, E. Townsend, M. Kabesch, S. K. Weiland, D. Carr, M. E. von, I. M. Adcock, P. J. Barnes, G. M. Lathrop, M. Edwards, M. F. Moffatt, and W. O. Cookson. 2003. Positional cloning of a novel gene influencing asthma from chromosome 2q14. Nat.Genet. 35:258-263.
27. Sano, K., H. Tanihara, R. L. Heimark, S. Obata, M. Davidson, J. T. St, S. Taketani, and S. Suzuki. 1993. Protocadherins: a large family of cadherin-related molecules in central nervous system. EMBO J. 12:2249-2256.
28. Holloway, J. W. and G. H. Koppelman. 2007. Identifying novel genes contributing to asthma pathogenesis. Curr.Opin.Allergy Clin.Immunol. 7:69-74.
29. Sekerel, B. E., Y. Saraclar, O. Kalayci, F. Cetinkaya, A. Tuncer, and G. Adalioglu. 1997. Comparison of four different measures of bronchial responsiveness in asthmatic children. Allergy 52:1106-1109.
30. Frank, M. and R. Kemler. 2002. Protocadherins. Curr.Opin.Cell Biol. 14:557-562.
31. Redies, C., K. Vanhalst, and F. Roy. 2005. delta-Protocadherins: unique structures and functions. Cell Mol.Life Sci. 62:2840-2852.
32. Hormi-Carver, K. K., W. Shi, C. W. Liu, and N. Berndt. 2004. Protein phosphatase 1alpha is required for murine lung growth and morphogenesis. Dev.Dyn. 229:791-801.
33. Fitsialos, G., A. A. Chassot, L. Turchi, M. A. Dayem, K. LeBrigand, C. Moreilhon, G. Meneguzzi, R. Busca, B. Mari, P. Barbry, and G. Ponzio. 2007. Transcriptional signature of epidermal keratinocytes

subjected to in vitro scratch wounding reveals selective roles for ERK1/2, p38, and phosphatidylinositol 3-kinase signaling pathways. J.Biol.Chem. 282:15090-15102.

34. Holgate, S. T., D. E. Davies, R. M. Powell, P. H. Howarth, H. M. Haitchi, and J. W. Holloway. 2007. Local genetic and environmental factors in asthma disease pathogenesis: chronicity and persistence mechanisms. Eur.Respir.J. 29:793-803.
35. Ohashi, Y., S. Motojima, T. Fukuda, and S. Makino. 1992. Airway hyperresponsiveness, increased intracellular spaces of bronchial epithelium, and increased infiltration of eosinophils and lymphocytes in bronchial mucosa in asthma. Am.Rev.Respir.Dis. 145:1469-1476.
36. Sandilands, A., A. Terron-Kwiatkowski, P. R. Hull, G. M. O'Regan, T. H. Clayton, R. M. Watson, T. Carrick, A. T. Evans, H. Liao, Y. Zhao, L. E. Campbell, M. Schmuth, R. Gruber, A. R. Janecke, P. M. Elias, M. A. van Steensel, I. Nagtzaam, M. van Geel, P. M. Steijlen, C. S. Munro, D. G. Bradley, C. N. Palmer, F. J. Smith, W. H. McLean, and A. D. Irvine. 2007. Comprehensive analysis of the gene encoding filaggrin uncovers prevalent and rare mutations in ichthyosis vulgaris and atopic eczema. Nat.Genet. 39:650-654.
37. Monsuur, A. J., P. I. de Bakker, B. Z. Alizadeh, A. Zhernakova, M. R. Bevova, E. Strengman, L. Franke, R. van't Slot, M. J. van Belzen, I. C. Lavrijsen, B. Diosdado, M. J. Daly, C. J. Mulder, M. L. Mearin, J. W. Meijer, G. A. Meijer, E. van Oort, M. C. Wapenaar, B. P. Koeleman, and C. Wijmenga. 2005. Myosin IXB variant increases the risk of celiac disease and points toward a primary intestinal barrier defect. Nat.Genet. 37:1341-1344.

SUPPLEMENTARY DATA

Method section

Study populations

Dutch asthma families and asthma trios. Two hundred families (1259 individuals) were ascertained through probands with asthma who were initially studied between 1962 and 1975 at Beatrixoord Hospital, Haren, The Netherlands (E1). Patients with symptomatic asthma who were not experiencing a current asthma exacerbation were referred to this hospital and admitted for a standardized, comprehensive evaluation for asthma and atopy. At the time of initial testing, all probands had asthma symptoms, were hyperresponsive to histamine (PC20 histamine $\leq$ 32 mg/ml), and were younger than 45 years of age. The families of two hundred of these original probands with their spouses, children, children's spouses, and grandchildren older than 6 years of age were recruited and evaluated between 1991 and 1999. Participants had been asked to stop their asthma and allergy medication if possible. They stopped inhaled corticosteroids for 14 days before testing, inhaled long acting beta-agonists and oral antihistamines 48 hours before testing, and inhaled short acting beta-agonists and anticholinergics 8 hours before testing. Therapy with oral corticosteroids was continued. Asthma diagnosis was based on an algorithm (E1). Standardized pulmonary function testing, bronchial responsiveness to histamine, and total serum IgE and skin tests were performed as described previously (Table E1). BHR was defined as a $PC_{20} \leq$ 32 mg/ml histamine using a 30-second inhalation protocol.

A second, independent asthma population of 407 patients with asthma has been ascertained through local hospitals and media appeals between 1998 and 2003 (E2). Available parents, sibs, spouses and children donated DNA to form trios (e.g. a proband and both parents) for genetic analyses. All probands have been characterized using the standardized study protocol used in the families (Table E2). PCDH1 SNPs were genotyped using the Massarray SNP genotyping platform (Sequenom, Inc). Family based association tests in the families and the trios were performed using FBAT (http///www.biostat.harvard.edu) (E3). FBAT uses phenotype information of the children, but not of the parents. Therefore, a case-control analysis of *PCDH1* SNPs was done in the probands and their age-matched unaffected spouses using a Chi square test as an additional, independent test for association. The Medical Ethics Committee of the University Hospital Groningen, the University of Maryland and

Wake Forest University School of Medicine approved this study, and all participants provided informed consent.

Vlagtwedde/Vlaardingen cohort study. The Vlagtwedde/Vlaardingen cohort is a general population-based cohort of Caucasian subjects in the Netherlands, which started in 1965 with a follow-up of over 25 years. In the last survey in 1989-1990, 2,467 subjects participated. Surveys were performed every three years, in which information was collected on respiratory symptoms, smoking status, FEV_1, and allergy skin tests (Table E2) (E4). At every visit, hyperresponsiveness to histamine was assessed in a random sample of this population, resulting in 418 subjects with one or more BHR measurement(s) over time and sufficient DNA. Bronchial hyperresponsiveness (BHR) was defined as a 10 % fall of forced expired volume in 1 second (FEV_1) at 16 mg / ml histamine, using a 30 seconds inhalation protocol. The association between *PCDH1* SNPs and the occurrence of becoming BHR positive over time was investigated. Data were analysed longitudinally using a Cox regression model (SPSS 14.0), with adjustment for smoking, age at investigation and FEV_1 at baseline. *PCDH1* SNPs were genotyped using primers and probes that were obtained from Applied Biosystems TaqMan® SNP Genotyping Assays using the Assay-by-Design service for which we provided sequences. The study protocol was approved by the local university hospital medical ethics committee and all participants gave their written informed consent

US children's respiratory study (Tucson, AZ, USA). A total of 1,246 healthy newborns and their nuclear families were enrolled between 1980 and 1984. More than three-fourths of these subjects (n=943) have been followed for the first 14 to 16 yr of life. Of this cohort, 513 unrelated enrollees who were still living in Tucson gave consent for genetic studies of asthma and allergies. Of these 513 subjects, 314 were non-Hispanic whites, 89 were Hispanics, 99 were mixed non-Hispanic whites/other (90% non-Hispanic white/Hispanic), and 11 were of other ethnicities. BHR was measured using nebulised cumulative dosages from 0.004 to 2.048 mg methacholine, until a fall of 20 % in FEV_1. Children were tested for BHR at age 11 and 16, and were excluded if experiencing an upper respiratory infection or using asthma medication in the previous three months (Table E2) (E5). Data was expressed as a methacholine dose response slope and differences between genotype groups were assessed using ANOVA. *PCDH1* SNPs were genotyped using standard methods. The Human Subjects Committee of the University of Arizona (Tucson, AZ) approved this study. Informed consent was obtained from parents of all subjects involved.

UK asthma families (Southampton). Three hundred forty one affected sib pair families from the UK were investigated, that have been investigated previously in identification of the *ADAM33* gene (E6). Asthma was defined as a physician's diagnosis of asthma and the report of current medication use. For the purpose of this analysis, a least squares slope measure of bronchial hyperresponsiveness was investigated using age and smoking status as covariates. Lung function and BHR testing were performed as described previously. Bronchial responsiveness was measured in subjects with a baseline FEV_1 of >70% predicted using inhaled methacholine. Passive smoking was assessed using a questionnaire response to questions on passive exposure to cigarette smoke in utero or in the first years of life (Table E2). *PCDH1* SNPs were genotyped using primers and probes that were obtained from Applied Biosystems TaqMan® SNP Genotyping Assays using the Assay-by-Design service for which we provided sequences. Family based association analyses were performed with FBAT (http///www.biostat.harvard.edu) (E3). Ethical approval for this work was granted by the Local Research Ethics Committees for each collection site and parental or individual informed consent obtained from all subjects.

US case control populations were recruited as described previously (E7). In brief, asthma cases and unaffected controls were recruited from three ethnically diverse US populations (African American, Hispanic, and Caucasian) as part of the Collaborative Study on the Genetics of Asthma (E8). Patients with asthma were defined by having a physician's diagnosis of asthma with BHR or bronchodilator reversibility; and reporting respiratory symptoms. Controls did not report symptoms of asthma, and were not tested for BHR or reversibility and did not have any first degree relatives with a diagnosis of asthma. Lung function testing, allergy skin tests, BHR measurements to methacholine or reversibility to albuterol were performed in the cases as described previously (Table E2) (E8). BHR was defined as a fall in baseline FEV_1 >20% after inhalation of <25 mg/ml methacholine. SNPs were genotyped using the Massarray SNP genotyping platform (Sequenom, Inc). Asthma susceptibility was tested in cases and controls using Chi square analysis, whereas BHR was tested using ANOVA in the cases only. This study was approved by the Institutional Review Board of Wake Forest University School of Medicine. Written informed consent for all adults, as well as written parental assent for children were obtained from all participants.

Genetic methods

Microsatellite marker screen

22 microsatellite markers were identified by resequencing the region of interest on NT_029289. Primers designed around repeat regions were used to screen for polymorphic markers and subsequently genotyped in the Dutch families, together with known markers (E9). Multiplex PCR was performed with fluorescently labeled primers. PCR products were separated on denaturing polyacrylamide gels, and the fragments were detected with ABI 377 sequencers (Perkin Elmer Applied Biosystems, Foster City, Calif). The fragments were scanned and scored with ABI software. A modified version of the program Linkage Designer was used to bin the alleles and check inheritance (E10). Genotyping errors, double recombinants, and inheritance inconsistencies were detected with the LINKAGE and CRIMAP software. Linkage analysis of the microsatellite fine screen markers on chromosome 5q31-33 was performed using GENEHUNTER-PLUS, as described previously (E9). Fine mapping markers were selected based on linkage disequilibrium through the computer package Merlin (r^2>0.1) to select on LD block. Microsatellite marker is available through NCBI databases and further information is available through the author upon request.

SNP screen

SNPs were selected in the region of interest from Hapmap using the tagger function of Haploview (r^2 =0.8, minor allele frequency of 5 %). Outside this region, additional SNPs were selected at 10 kb intervals to further characterize the region. SNPs were genotyped using the Massarray SNP genotyping platform (Sequenom, Inc). PCR and extension primers were designed by using Spectrodesigner software (Sequenom, Inc), and reactions were performed according to the manufacturers instructions. The genotyped SNPs are listed in Table E3. Genotyping was performed blinded from the clinical data. Quality control of the SNP screen included measures of Hardy Weinberg equilibrium, with all markers that were out of HWE (p value < 0.01) being removed. Moreover, the family design enabled us to observe any Mendelian errors that may have been due to low genotyping quality.

Resequencing of the PCDH1 gene

The *PCDH1* gene was resequenced in 96 subjects that have been described previously (E11): 16 cases and 8 controls from four different populations: Dutch, US African Americans, US Hispanics and US Caucasians. Overlapping PCR fragments ~ 400-600 bp were generated to sequence approximately 2 kb of the putative promoter region, all exons, and the complete 3'

untranslated region (UTR) regions. Each 30 µl PCR contained 30 ng of genomic DNA, 1x PCR buffer (Life Technologies, Gaithersburg, MD, USA), 1.5 mM dNTPs, 1.5 mM MgCl2, 200 mM dNTPs, 15 pmoles of each forward and reverse primer, and 0.5 U of Taq polymerase. Depending on prior reaction optimization, general cycling conditions were: 94 °C 4 min, followed by 25-30 cycles at 94 °C for 1min, Tanneal for 1 min, and at 72 °C for 1 min; and finishing with a single extension cycle at 72 °C for 5 min. PCR products were purified using the Quick step 96 well PCR purification kit (Edge Biosystems, Gaithersburg, MD, USA) and stored in water at – 20 ° C. DNA sequencing was performed using ABI Big Dye Terminator sequencing kit (Applied Biosystems, Inc., Foster City, CA, USA). Each sequencing reaction contained 10 – 50 ng of purified PCR product, 1.5 pmoles of sequencing primer, 1 mmol of BigDye Terminator mix, 1.5 µl of 5X sequencing dilution buffer (400 mM Tris pH 9.0, 10 mM MgCl2) and water to volume. Cycling conditions were 94 °C for 1 min; 25 cycles at 94 °C for 30 sec, 50 °C for 30 sec, and 60 °C for 4 min; and finishing with a single 72 °C extension step for 5 min. Sequencing products were ethanol precipitated, air dried, resuspended in 10 –25 l of ddH_2O, and analyzed on an ABI 3700 DNA analyzer. Data was aligned and polymorphisms were detected using Sequencher DNA analysis software (Gene Codes Corporation, Ann Arbor, MI, USA).

PCDH1 mRNA expression. The human bronchial epithelial cell line 16HBE 14o- (16HBE) was provided by Dr. D. C. Gruenert (University of California, San Francisco, CA) and cultured as described previously (E12). Epithelial cells were obtained through fiberoptic bronchoscopy by epithelial brushings from asthma patients, and were cultured for three passages as described previously (E13). After three passages mRNA and protein were isolated. Other cells were obtained from healthy donors or from autopsy material. Total RNA was isolated from 5 to 10 million cells by using the RNeasy® mini kit (Qiagen) as described by the manufacturor. Next 2 µg of RNA was reverse transcribed into cDNA via Omniscript® RT reaction (Qiagen), resulting in equal amount of cDNA. Generally 25 ng to 50 ng of cDNA was used as template for PCR. Two PCR reactions were performed for detecting PCDH1 on RNA level. The first PCR detects both predicted isoforms, the second only the 5 exon isoforms. First PCR of exon 2 towards exon 3 was performed with forward primer: 5'-TTCGCCTCACCAGTCATCACT-3', and reverse primer: 5'-TTGCTGTCACTGCCTGTCTCA-3'. The second PCR from exon 3 towards exon 5 was performed with forward primer: 5'-ACCTGCCACCTGCAAACACAT-3', and reverse primer TGGTAAGGCACGAAGGTGGAGA-3'. As housekeeping/control gene GAPDH was used

(forward primer: 5'-CGGAGTCAACGGATTTGGTCGTAT-3', reverse primer: AGCCTTCTCCATGGTGGTGAAGAC-3'). PCRs were performed by using Expand High Fidelity PCR System (Roche).

PCDH1 protein expression. For western blotting, confluent layers of 16HBE or primary epithelial cells (~5 * 10^6 cells) were lysed and mixed with protein sample buffer. Protein samples were separated on 7.5% or 10% acrylamide SDS-page gels (Bio-Rad Laboratories). Separated protein samples were transferred to PVDF-membranes (0.2µm pore-size, Bio-Rad Laboratories) by means of wet blotting procedure (Mini Trans-Blot cell, Bio-Rad Laboratories). Detection of PCDH1 on PVDF membranes was performed by applying two primary antibodies, one polyclonal, generated from immunized rabbit against 12 amino-acid peptide (DIAGDPEYERSK, Eurogentec, Liege, Belgium), and one monoclonal (H00005097-M01, 100 mg, Abnova, Taiwan). As secondary antibodies goat anti rabbit conjugated to peroxidase (GARPO) and Peroxidase-conjugated rabbit-anti-mouse secondary antibody (RAMPO) were used, as tertiary antibodies rabbit anti goat conjugated to peroxidase (RAGPO) and GARPO were applied (Dako Cytomation, Denmark). Finally PCDH1 signal was detected by applying Luminol reagent (Fluka Biochemika) on membranes, and exposing membranes to Medical X-Ray films (Fuji Photo Film Gmbh, Germany).

Lung tissue samples and airway wall biopsies were embedded in paraffin. Immunohistochemistry was performed using the affinity purified polyclonal antibody (Eurogentec, Liege, Belgium). As secondary step, GARPO (Daco, Heverlee, Belgium), and as tertiary step RAGPO (Dako, Heverelee, Belgium) was used. Subsequently, AEC (3 amino – 9 - ethylcarbazole (Merck, Darmstadt, Germany) was applied as a chromogen and counterstained with haematoxylin (Merck, Darmstadt, Germany). Similar concentrations of rabbit IgG isotype antibodies were used as control. PCDH1 expression could be completely blocked by peptide preincubation (data not shown). Medical ethics committee approval and informed consent was obtained from all patients.

SUPPLEMENTARY REFERENCES

E1. Panhuysen, C. I., E. R. Bleecker, G. H. Koeter, D. A. Meyers, and D. S. Postma. 1998. Characterization of obstructive airway disease in family members of probands with asthma. An algorithm for the diagnosis of asthma. *Am.J.Respir.Crit Care Med.* 157:1734-1742.

E2. Jongepier, H., G. H. Koppelman, I. M. Nolte, M. Bruinenberg, E. R. Bleecker, D. A. Meyers, G. J. te Meerman, and D. S. Postma. 2005. Polymorphisms in SPINK5 are not associated with asthma in a Dutch population. *J.Allergy Clin.Immunol.* 115:486-492.

E3. Horvath, S., X. Xu, and N. M. Laird. 2001. The family based association test method: strategies for studying general genotype--phenotype associations. *Eur.J.Hum.Genet.* 9:301-306.

E4. van Diemen, C. C., D. S. Postma, J. M. Vonk, M. Bruinenberg, J. P. Schouten, and H. M. Boezen. 2005. A disintegrin and metalloprotease 33 polymorphisms and lung function decline in the general population. *Am.J.Respir.Crit Care Med.* 172:329-333.

E5. Stein, R. T., C. J. Holberg, W. J. Morgan, A. L. Wright, E. Lombardi, L. Taussig, and F. D. Martinez. 1997. Peak flow variability, methacholine responsiveness and atopy as markers for detecting different wheezing phenotypes in childhood. *Thorax* 52:946-952.

E6. Van Eerdewegh. P., R. D. Little, J. Dupuis, R. G. Del Mastro, K. Falls, J. Simon, D. Torrey, S. Pandit, J. McKenny, K. Braunschweiger, A. Walsh, Z. Liu, B. Hayward, C. Folz, S. P. Manning, A. Bawa, L. Saracino, M. Thackston, Y. Benchekroun, N. Capparell, M. Wang, R. Adair, Y. Feng, J. Dubois, M. G. FitzGerald, H. Huang, R. Gibson, K. M. Allen, A. Pedan, M. R. Danzig, S. P. Umland, R. W. Egan, F. M. Cuss, S. Rorke, J. B. Clough, J. W. Holloway, S. T. Holgate, and T. P. Keith. 2002. Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness. *Nature* 418:426-430.

E7. Basehore, M. J., T. D. Howard, L. A. Lange, W. C. Moore, G. A. Hawkins, P. L. Marshik, M. S. Harkins, D. A. Meyers, and E. R. Bleecker. 2004. A comprehensive evaluation of IL4 variants in ethnically diverse populations: association of total serum IgE levels and asthma in white subjects. *J.Allergy Clin.Immunol.* 114:80-87.

E8. Xu, J., D. A. Meyers, C. Ober, M. N. Blumenthal, B. Mellen, K. C. Barnes, R. A. King, L. A. Lester, T. D. Howard, J. Solway, C. D. Langefeld, T. H. Beaty, S. S. Rich, E. R. Bleecker, and N. J. Cox. 2001. Genomewide screen and identification of gene-gene interactions for asthma-susceptibility loci in three U.S. populations: collaborative study on the genetics of asthma. *Am.J.Hum.Genet.* 68:1437-1446.

E9. Meyers, D. A., D. S. Postma, O. C. Stine, G. H. Koppelman, E. J. Ampleford, H. Jongepier, T. D. Howard, and E. R. Bleecker. 2005. Genome screen for asthma and bronchial hyperresponsiveness: interactions with passive smoke exposure. *J.Allergy Clin.Immunol.* 115:1169-1175.

E10. Van Camp, G., W. Balemans, and P. J. Wellems. 2007. Linkage designer and linkage reporter software for automated gene localization studies. *Trends Genet* 1997 (13):82.

E11. Hawkins, G. A., P. J. Amelung, R. S. Smith, H. Jongepier, T. D. Howard, G. H. Koppelman, D. A. Meyers, E. R. Bleecker, and D. S. Postma. 2004. Identification of polymorphisms in the human glucocorticoid receptor gene (NR3C1) in a multi-racial asthma case and control screening panel. *DNA Seq.* 15:167-173.

E12. Heijink, I. H., P. M. Kies, H. F. Kauffman, D. S. Postma, A. J. van Oosterhout, and E. Vellenga. 2007. Down-regulation of E-cadherin in human bronchial epithelial cells leads to epidermal growth factor receptor-dependent Th2 cell-promoting activity. *J.Immunol.* 178:7678-7685.

E13. Borger, P., H. F. Kauffman, J. Scholma, J. A. Timmerman, and G. H. Koeter. 2002. Human allogeneic CD2+ lymphocytes activate airway-derived epithelial cells to produce interleukin-6 and interleukin-8. Possible role for the epithelium in chronic allograft rejection. *J.Heart Lung Transplant.* 21:567-575.

Supplementary Figures and Tables

Figure E1. *Multipoint point linkage analysis of microsatellite fine map on chromosome 5q31-33 in 200 Dutch families (all); 95 families ascertained through a proband with asthma and a significant cigarette smoking history of > 5 pack years ('smoke exposed') ; and 105 families not exposed to cigarette smoke. HLOD scores were generated using Genehunter using the genetic model as described previously (E11).*

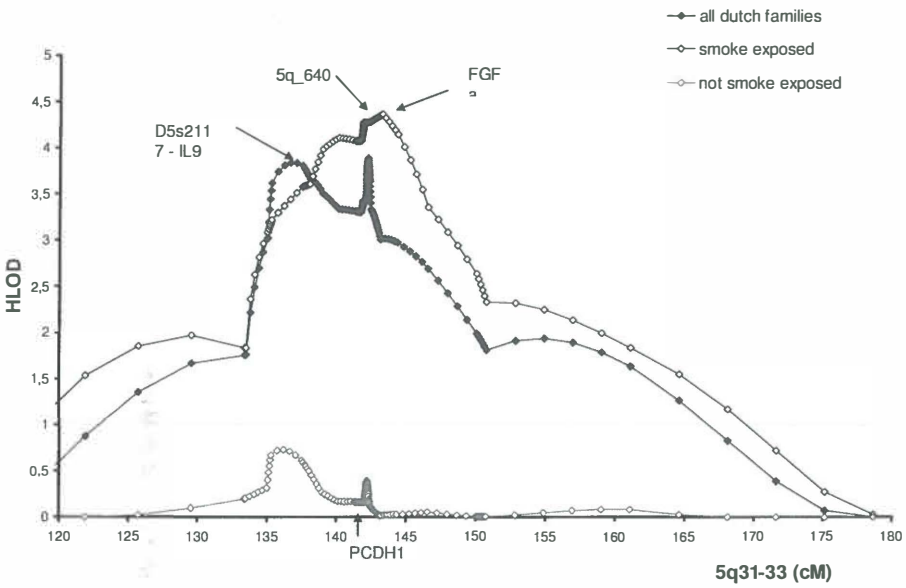


Table E1: Clinical characteristics of 200 Dutch families of probands with asthma.
** Mean values with range. Total IgE: geometric mean. # One proband married twice, both spouses participated in the study. Prebr= prebronchodilator. All probands were hyperresponsive at initial testing. Asthma was defined according to an algorithm (class I and II) (E1).*

	Probands	Spouses	Children	Grandchildren
Number	200	201 #	540	186
Age*, years	52.1 (8.4)	51.0 (9.2)	24.3 (9.2)	12.1 (5.6)
Male, %	62.0	37.8	45.0	49.3
Total IgE*, kU/l	92.9	26.2	64.1	62.4
	(1.0 - 2880)	(0.5 - 1940)	(0 - 3360)	(0.5 - 3710)
Positive skin test	81.9	31.0	54.1	38.0
FEV_1%predicted prebr	69.6	98.4	93.7	90.5
FEV_1%VC prebr, %	59.7	76.7	81.0	82.6
BHR, %	88.2	25.6	46.2	59.6
Asthma, %	100	10.6	31.5	39.2
≥ 5 pack years smoking,%	47.5	58.2	24.6	7.3

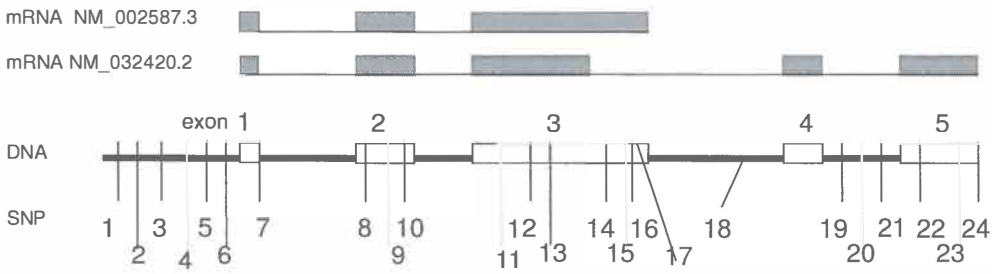
Table E2: Clinical characteristics of the replication cohorts
*Legend. Means and percentages are presented. Dutch asthma trios. All patients had a doctor's diagnosis of asthma, 86.9 % was classified as class I or II according to an algorithm. Dutch general population: Data was presented for last visit (1989/1990), apart from BHR data, that was taken from any visit where BHR measurements were performed. BHR was defined as PC10 ≤ 16 mg /ml. * In UK families, BHR was reported as FEV_1 response to methacholine (transformed to 1/(least squares slope+30) X 1,000; BHR = Bronchial Hyperresponsiveness, Quest = Questionnaire. # In US case control populations, 757 controls (Caucasian, n = 327; Hispanic, n = 130 and Afro American n = 300) were defined as having no symptoms of asthma and a negative family history. These controls did not undergo clinical testing.*

	Dutch asthma trio probands	Dutch general population	US Tucson (Age 11)	UK Southampton families (children)	US Caucasian Cases #	US Hispanic Cases #	US Afro-Am. Cases #
N	407	418	318	822	338	116	222
Age, years	33.8	51.7	10.7	11.4	27.1	17.4	24.4
Male, %	36.9	54.8	48.2	53.6	37.9	46.5	40.5
Asthma, %	86.9	N. / A.	9.7	87.2	100	100	100
BHR, %	91.0	51.4	25.6	14.6 *	89.7	91.4	69.8
FEV_1 (% pred.)	87.4	96.3	100.4	95.7	81.4	95.2	75.4
Pos. skin test, %	82.9	13.6	65.1	61.3	90.6	85.1	90.4

Table E3: SNPs (name and chromosomal location on chromosome 5 built 36.3, NT_029289.10) genotyped on chromosome 5q in 200 Dutch asthma families

rs2530223	140994677	rs2974703	141205502	rs3747717	141301822
rs456998	141005345	rs17208551	141207154	rs164083	141305821
rs6580194	141014306	rs17097812	141209072	rs2434322	141315105
rs4912610	141034423	rs7719391	141209824	rs164073	141315425
rs349132	141044810	rs3935792	141212177	rs164075	141316448
rs6580201	141052668	rs7711783	141215263	rs164077	141317409
rs349128	141061080	rs11745262	141216080	rs252108	141318955
rs248616	141071494	ivs3-116	141217333	rs3761762	141319011
rs755772	141081269	rs12523337	141217532	rs252109	141319072
rs9686896	141091090	rs11167761	141218526	rs164078	141319164
rs32926	141099223	rs12653948	141222067	rs164079	141319947
rs32946	141109387	rs14359	141222817	rs6580209	141324307
rs11738818	141118664	rs3797054	141223830	rs252137	141327014
rs740666	141128665	rs3822357	141224540	rs740662	141336132
rs152355	141136482	rs10063472	141226830	rs252101	141344936
rs248492	141146058	rs10054186	141226998	rs364287	141347991
rs457639	141151926	rs12515587	141229146	rs433623	141348082
rs17610222	141153511	rs4563655	141231724	rs409037	141354387
rs152267	141157045	rs12517246	141232232	rs252114	141362768
rs1428726	141162888	rs6888135	141234246	rs108593	141366822
rs2974706	141167646	rs6580203	141237242	rs164080	141371716
rs17097654	141168499	rs10057157	141238949	rs3910558	141377510
rs187454	141171907	rs13355929	141239217	rs492620	141394644
rs17648891	141176516	rs4912782	141242610	rs7722161	141406068
rs17648933	141181221	rs17097839	141258742	rs6580216	141413259
rs17610605	141181524	rs9324859	141259534	rs1438699	141422317
rs17610619	141182387	rs6862407	141266527	rs252215	141431021
rs17525960	141184044	rs4141206	141276686	rs4912800	141437879
rs248514	141185129	rs10035631	141277013	rs2338874	141447110
rs17705472	141186045	rs4912787	141277307	rs891990	141455645
rs13162099	141186515	rs3855086	141279161	rs735683	141465352
rs248504	141189651	rs3861854	141280553	rs17287176	141483936
rs166040	141190936	rs445310	141281090	rs2043280	141493875
rs400540	141197958	rs917531	141285467	rs1062158	141503184
rs3815355	141200189	rs351260	141290008	rs449454	141513246
rs1709780	141202437	rs2286523	141294434	rs17705913	141521095
				rs6890366	141531043

Table E4: Protocadherin-1: Gene, mRNA expression and SNP position.
NT_ AC094107.3: position on reference contig NT_ AC094107.3. Cauc. US Caucasian; Afr Am US African American, Hisp. US Hispanic. Np not polymorphic. SNPs in bold were selected for genotyping in complete study populations.



SNP	region	name	AC094107.3	rs number	Allelel 1	Allele 2	Frequency (allele 2) in sequencing panel			
							Cauc.	Afr Am	Hisp.	Dutch
1	promoter	-1440	92385		A	C	np	0.02	np	np
2	promoter	-1362	92307		A Ins	A Del	np	0.02	np	np
3	**promoter**	**-1206**	**92151**	**rs13355929**	**G**	**A**	**0.05**	**0.41**	**np**	**0.02**
4	**promoter**	**-938**	**91883**	**rs10057157**	**G**	**A**	**0.45**	**0.13**	**0.60**	**0.39**
5	promoter	-924	91869		C	T	np	0.02	np	np
6	promoter	-909	91854		TT Ins	TT Del	np	0.02	np	np
7	intron 1	IVS1 -26	82140		C	T	0.04	np	np	0.05
8	exon 2	Leu15Phe	82112	rs12517385	C	T	np	np	0.02	0.02
9	**exon 2**	**His25Pro**	**82081**	**rs12515587**	**A**	**C**	**0.11**	**0.80**	**0.10**	**0.17**
10	exon 2	Ala287Ala	81294		C	T	0.02	0.04	np	np
11	**exon 3**	**Ala514Thr**	**77474**	**rs3822357**	**G**	**A**	**0.04**	**0.02**	**0.08**	**np**
12	exon 3	Asn601Lys	77211		T	G	np	0.04	np	np
13	exon 3	Ala712Thr	76880	rs34636888	G	A	np	np	np	0.03
14	**exon 3**	**Ala750Ala**	**76764**	**rs3797054**	**T**	**C**	**0.35**	**0.37**	**0.34**	**0.25**
15	exon 3	Gly903Ser	76307		G	A	np	0.02	np	np
16	exon 3	Gly1013Gly	75975		C	G	0.04	np	np	0.02
17	**exon 3**	**IVS3 +164**	**75751**	**rs14359**	**G**	**C**	**0.19**	**0.09**	**0.13**	**0.25**
18	**intron 3**	**IVS3 -116**	**70270**		**TTC Ins**	**TTC Del**	**0.09**	**0.02**	**0.10**	**0.08**
19	intron 4	IVS4 +50	69885		G	A	0.03	np	np	np
20	intron 4	IVS4 +152	69783		T	C	0.03	np	np	np
21	intron 4	IVS4 +233	69702		C	T	np	0.02	np	np
22	exon 5	Ala1149Ala	66992		C	T	0.02	np	np	np
23	3' UTR	3'UTR +43	66682		C	T	np	np	0.04	np
24	3' UTR	3'UTR+238	66487	rs10213790	G	T	np	0.06	np	np

Table E5: Interaction of PCDH1 gene variants and environmental tobacco smoke exposure in utero / first years of life

** Data not shown due to differences in allele frequency of Hispanic and Caucasian populations*

SNP	Risk allele	Dutch Asthma families		Dutch Asthma trios		Tucson, US General population, children		UK asthma families	
						At age 11	at age 16		
		ETS	No ETS	ETS /	No ETS	ETS / No ETS	ETS / No ETS	ETS / No ETS	
N		635	624	944	277	58/215	50/233	1508	1508
Analysis		FBAT	FBAT	FBAT	FBAT	ANOVA	ANOVA	FBAT	FBAT
Ala514Thr	G	0.35	0.09	0.89	0.53	0.4 / 0.06	0.33 / 0.1	0.009	0.09
Ala750Ala	T	0.18	0.01	0.88	0.24	0.078 / 0.125	0.9 / 0.035	0.19	0.68
IVS3+164	C	-	-	0.58	0.19	0.79 / 0.70	0.75 / 0.88	0.18	0.41
IVS3 -116	Del TTC	0.20	0.047	0.09	0.37	*	*	0.58	0.89

Chapter 3

Protocadherin-1 polymorphisms are associated with eczema in two Dutch birth cohorts

Henk Koning
Dirkje S. Postma
Bert Brunekreef
Eric J. Duiverman
Henriette A. Smit
Carel Thijs
John Penders
Marjan Kerkhof
Gerard H. Koppelman

Pediatr Allergy Immunol. 2012. Vol 23(3): p270-277

ABSTRACT

Background: Eczema and asthma share a common genetic background and show linkage to chromosome 5q31-33. Protocadherin-1 (*PCDH1*) is located in this region and was identified as a susceptibility gene for bronchial hyperresponsiveness (BHR), a hallmark of asthma. *PCDH1* encodes an adhesion molecule, expressed in airway and skin epithelium.

We determined whether *PCDH1* polymorphisms, previously associated with asthma or BHR, also associated with questionnaire and UK Working Party (UKWP) defined eczema.

Methods: Four *PCDH1* polymorphisms were genotyped in two Dutch birth cohorts, PIAMA (n=967) and KOALA Birth Cohort Study (n=1560). Association with eczema was determined by Chi-square tests, and Generalized-Estimating-Equations (GEE).

Results: Insertion deletion IVS3-116 was associated with development of UKWP-eczema in PIAMA (age 4, OR=1.90 (1.14-3.18)), and borderline with questionnaire-reported eczema in PIAMA (GEE, OR=1.33 (0.98-1.81)). Furthermore IVS3-116 was associated with questionnaire-reported eczema in KOALA (age 1, OR=1.44 (1.00-2.07)). Pooled analysis of questionnaire-reported eczema of both cohorts resulted in a significant association of IVS3-116 with eczema (OR=1.26 (1.01-1.58)). Rs3822357 (A-allele) associated with protection for eczema in PIAMA only (questionnaires, OR=0.19 (0.06-0.63)).

Conclusions: PCDH1 gene variant IVS3-116 associates with eczema in two independent birth cohorts. Combined with previous observations this indicates a shared genetic susceptibility to BHR, asthma and eczema.

INTRODUCTION

Atopic diseases such as eczema and asthma are increasingly prevalent in the western world (1). These complex diseases are caused by an interplay of genetic and environmental factors (2). Several prospective studies have shown that eczema pre-dates or co-exists with asthma (3, 4). Twin studies indicate that these atopic diseases share a common genetic background, but that also disease specific genetic factors exist (5).

The chromosomal region 5q31-33 is strongly linked to eczema (6, 7), asthma and bronchial hyperresponsiveness (BHR) (8-10). In this region, multiple atopy susceptibility genes have been identified, such as *SPINK5* (eczema), *IL-13* (asthma, atopy) and *CD14* (atopy). One gene in this region, *Protocadherin-1* (*PCDH1*), has recently been discovered as a BHR susceptibility gene (11).

PCDH1 is thought to play a role in cell-cell adhesion (12, 13). PCDH1 mRNA and protein expression was identified in airway epithelium (11), whereas PCDH1 mRNA is expressed in skin keratinocytes (14, 15). A defect in epithelial barrier function may represent an underlying common mechanism in asthma, BHR, and eczema (16).

We hypothesized that *PCDH1* gene variants may represent a common genetic background of eczema and asthma, due to its proposed barrier function in epithelial cells. We therefore determined whether SNPs, that were shown previously to associate with asthma and BHR, are associated with eczema. To this aim we investigated the association of *PCDH1* SNPs with eczema in two Dutch prospective birth cohorts.

MATERIAL AND METHODS

Study populations

Two Dutch populations were investigated: 1) The Prevention and Incidence of Asthma and Mite Allergy (PIAMA) birth cohort, 2) KOALA (Dutch acronym for: Child, Parent, health, Focus on Lifestyle and Predisposition) birth cohort. The local medical ethics committees approved these studies, and all participants provided written (parental) informed consent.

Ascertainment

Recruitment of subjects for PIAMA birth cohort – Study design of the PIAMA birth cohort has been described previously (17). In summary, recruitment took place during the first trimester of pregnancy with a validated screening questionnaire (18) and was conducted by 52 midwife practices in 3 different regions in the Netherlands: north (Groningen and surroundings), central (Bilthoven and Wageningen and surroundings), and southwest (Rotterdam and surroundings). Women reporting any of the following symptoms were defined as allergic (*i.e.* a history of asthma, current hay fever, current allergy to house dust mite, or pets) and their children were defined as 'high-risk'. A total of 10,232 pregnant women completed the questionnaire and 2,949 (29%) of them were 'allergic'. The participating children were born between May 1996 and December 1997, and were followed yearly, up to 8 years of age.

At baseline, the PIAMA study population consisted of 4,146 children, 183 (5%) were lost to follow-up. The study therefore started with 3963 newborns: 1,327 children from allergic mothers and a random sample of 663 children from non-allergic mothers were selected for medical examinations, from which 1,808 children were eligible for a medical examination at age 4, and of whom 1,288 children participated. DNA was collected at age 4 from 1037 children during medical examinations for atopic eczema. Children of non-Dutch mothers were excluded from the analysis. At the age of 1 year medical examination for eczema was performed only for the intervention group (n=414). Intervention consisted of mite-impermeable mattress covers. Study population characteristics are described in Table 1.

Recruitment of subjects for the KOALA cohort - The design of the study has been described previously (19). Briefly, participants with diverse lifestyles (conventional and alternative) were recruited at 34 weeks of gestation. Pregnant women with a conventional lifestyle (n=2343) were recruited from an ongoing prospective cohort study on Pregnancy-related Pelvic Girdle pain in the Netherlands. Additionally, pregnant women with an alternative lifestyle (n=491) were recruited through organic food shops, anthrophosophic doctors and midwifes, Steiner Schools, and magazines. During the first two years postpartum, and at the age of 6 years information on atopic outcomes and their determinants were collected for all members of the cohort by repeated questionnaires at ages 3, 7, 12, 24 months and 6 years. The study was approved by the medical ethics committee of the Maastricht University. Written informed parental consent was obtained from all participants. All participants were asked for providing DNA via buccal swaps (n=1,560). Dutch children were

medically examined for atopic eczema at the age of 2 years only (n=676). Characteristics of the study population are described in Table 1.

Clinical evaluation

Diagnosis of eczema. For the PIAMA cohort at the age of 1 to 8 years, and for the KOALA cohort at 7, 12, 24 months, and 6 years, infants were defined as having developed eczema if their parents reported eczema based on ISAAC (the International Study of Asthma and Allergies in Childhood) questions. The ISAAC questionnaire defines eczema as 'the presence of a history of itchy rash which was coming and going in the last 12 months, localized on flexural sites on folds of the elbows, behind the knees, in front of the ankles, under the buttocks, or around the neck, ears or eyes. Cases of only diaper rash, rash around the eyes and/or scalp scaling were excluded.

At home visits at the age of 1 and 4 years for PIAMA and at 2 years for KOALA, trained health nurses examined infants for atopic dermatitis according to the UK Working Party (UKWP) criteria (20-22) in a subset of the population. In PIAMA, home visits were performed at 1 year for children of the intervention study, and at 4 years for a random sample of the cohort. In PIAMA, a mandatory modification was made to the UKWP – criteria at 1 year only, since the UKWP criteria "early onset" and "presence of other atopic diseases" are less relevant for children at 12 months of age (23).

In KOALA, home visits were performed in children whose parents gave permission to draw blood samples. Infants with a UKWP probability score (20) of atopic dermatitis (AD) > 90% were regarded as infants with 'probable presence of atopic dermatitis' as was determined previously (24).

Diagnosis of asthma: In the PIAMA cohort we analysed 'doctor-diagnosed asthma', defined as asthma ever diagnosed by a doctor and asthma present in the last 12 months. Second, we used a definition of asthma based on symptoms, defined as at least one attack of wheeze or dyspnoea and/or the prescription of inhaled corticosteroids in the last 12 months (25). In PIAMA bronchial hyperresponsiveness at 8 years was defined as a fall of ≥20% in FEV1 after inhalation of a maximum of 0.62 mg methacholine bromide (26). In the KOALA cohort asthma at the age of six years was defined by asthma symptoms: at least one attack of wheezing or dyspnoea, or regular use of inhaled asthma medications in the last year.

Table 1: Participant characteristics for PIAMA and KOALA study populations and total birth cohorts

Characteristics	PIAMA Study Population	PIAMA Total Cohort	KOALA Study Population	KOALA Total Cohort
Number	967	3,963	1,560	2,834
Ethnicity (% Dutch origin)	100	94.2	100	95.8
Boys (%)	51.9	51.8	50.3	51.3
Education level mother (%)				
Low	18.3	23.5	8.3	10.7
Intermediate	42.7	41.5	37.3	39.4
High	39.0	35.0	54.4	49.9
Family history (%)				
Atopy mother	65.9	31.3	32.9	34.1
Asthma mother	16.3	7.9	8.9	9.3
Atopy father	31.7	30.8	36.6	35.0
Asthma father	7.3	7.7	10.1	10.0
Children at risk (%)[1]	75.6	51.5	55.8	56.5
Intervention (type)	Mattress covers	Mattress Covers	No	No
No (%)	57.1	80.7	100	100
Placebo (%)	24.3	10.0	0	0
Active (%)	18.6	9.3	0	0
Environmental exposures				
Breast feeding (%)				
Never	14.6	17.9	14.6	19.8
< 3 months	35.0	38.4	20.7	21.1
≥ 3 months	50.4	43.7	64.7	59.1
ETS at home first year (%)	22.9	27.8	11.5	15.4
Pet (dog and/or cat) first year (%)	39.6	43.2	39.8	41.9
Dog first year (%)	14.3	16.1	19.8	22.2
Cat first year (%)	29.1	32.8	24.3	23.8
Presence older siblings at birth (%)	48.8	50.5	58.3	55.7
Eczema manifestations				
ISAAC questionnaire				
1 year (%)	18.5; n=174	15.2; n=559	18.0; n=263	19.4; n=484
2 years (%)	20.1; n=189	16.6; n=616	15.4; n=234	15.3; n=389
3 years (%)	22.3; n=209	17.3; n=632	N.a.	N.a.
4 years (%)	21.5; n=200	17.9; n=631	N.a.	N.a.
5 years (%)	17.9; n=164	15.3; n=515	N.a.	N.a.
6 years (%)	18.0; n=165	15.4; n=527	13.6; n=177	13.9; n=275
7 years (%)	16.9; n=150	14.3; n=479	N.a.	N.a.
8 years (%)	18.3; n=159	16.0; n=515	N.a.	N.a.
UKWP criteria				
1 year (%)	13.7; n=56	14.3; n=479	N.a.	N.a.
2 years (%)	N.a.	N.a.	13.8; n=93	13.4; n=116
4 years (%)	12.0 ; n=115	12.2; n=155	N.a.	N.a.
Total serum IgE				
1 year (IU/ml)[2]	7.1 (2.0-17.0) n=346	6.6 (2.0-17.0) n=565	6.0 (2.6-12.4) n=644	6.4 (2.7-13.3) n=912
2 years (IU/ml)[2]	N.a.	N.a.	12.0 (3.6-38.0) n=658	12.2 (3.8-38.5) n=829
4 years (IU/ml)[2]	35.0 (12.0-95.0) n=664	37.3 (13.0-107.3) n=746	N.a.	N.a.
8 years (IU/ml)[2]	65.0 (23.0-230) n=703	62.8 (20.0-227.5) n=1,713	N.a.	N.a.
Asthma manifestations				
Asthma symptoms				
3 years (%)	25.3; n=245	22.9; n=844	N.a.	N.a.
4 years (%)	24.0; n=230	18.8; n=670	N.a.	N.a.

5 years (%)	22.1; n=211	17.6; n=615	N.a.	N.a.
6 years (%)	18.3; n=174	14.6; n=507	11.2; n=146	11.6; n=228
7 years (%)	16.6; n=151	12.3; n=416	N.a.	N.a.
8 years (%)	17.2; n=158	13.0; n=429	N.a.	N.a.
Doctor-diagnosed asthma				
1 year (%)	7.2; n=69	6.1; n=224	N.a.	N.a.
2 years (%)	5.6; n=54	4.8; n=177	N.a.	N.a.
3 years (%)	5.1; n=49	4.1; n=152	N.a.	N.a.
4 years (%)	5.2; n=50	4.1; n=145	N.a.	N.a.
5 years (%)	5.1; n=48	4.0; n=138	N.a.	N.a.
6 years (%)	5.8; n=55	3.9; n=134	N.a.	N.a.
7 years (%)	4.2; n=38	2.8; n=98	N.a.	N.a.
8 years (%)	5.1; n=45	3.6; n=116	N.a.	N.a.
Bronchial hyperresponsiveness				
8 years (%)	44.9; n=293	42.9; 402	N.a.	N.a.

Abbreviations: n = number of samples available; N.a. = not available. Definitions of characteristics were described previously (27).

[1] *Defined as: children who have at least one parent with asthma or atopy.*

[2] *Geometric mean (interquartile range).*

Genotyping

Subjects from the PIAMA and KOALA cohorts were genotyped for three *PCDH1* single nucleotide polymorphisms (SNPs) (rs3797054, rs3822357, and rs14359) and one insertion deletion (IVS3-116, Figure 1A), in amplified DNA by Competitive Allele-Specific PCR using KASPar™ genotyping chemistry, performed under contract by K-Biosciences, as described previously (27). These polymorphisms were selected based on previous associations of *PCDH1* with BHR and asthma (11).

Linkage Disequilibrium (LD) was calculated using Haploview v4.1, by determining D' and r^2 values (http://www.broad.mit.edu/mpg/haploview). Genotyping was performed blinded of the clinical status and considered successful when less than 5% of the genotypes were missing. Furthermore, all SNPs were tested for deviations from Hardy Weinberg equilibrium, using χ^2-tests ($p>0.05$).

Statistical analysis

Eczema was investigated for association with *PCDH1* gene variants using chi-squared tests, comparing genotype frequencies between affected and non-affected individuals. Odds Ratios (OR) and 95% confidence intervals (95% CI) were calculated from logistic regression analysis. To detect a possible influence of asthma on the associations of *PCDH1* with eczema, analyses were repeated adjusted for asthma.

Generalized Estimating Equations (GEE) were used to assess associations between *PCDH1* gene variants and the presence of eczema in the first 8 years of life. GEE is a longitudinal statistical technique that takes correlations between repeated measurements in the

same individual into account. Since 8 observations on atopic dermatitis were made on the same subject, we performed a GEE analyses to analyse the relationships of *PCDH1* SNPs with eczema simultaneously. A 7-dependent working correlation structure fitted the data best. This assumes that the correlations t measurements apart are equal, the correlations t+1 measurements apart are equal and so on for t=1 to t=7. We present the overall estimates based on yearly measurements. We repeated GEE analysis adjusted for parental atopy, sex, breastfeeding, intervention and study population, and inspected whether these covariates alter the regression coefficient ≥ 10%. In addition analyses were repeated adjusted for asthma. We previously investigated and reported the validity of pooling data from these two highly comparable birth cohorts (27). For all statistical analysis we report associations obtained by the best-fitting genetic model.

RESULTS

Genotyping

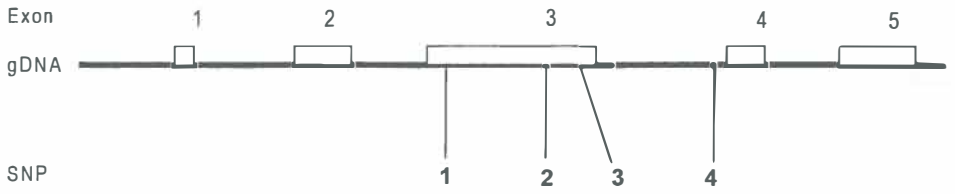
All tested SNPs were located in or nearby exon 3 of *PCDH1.* All SNPs were in Hardy Weinberg equilibrium (p>0.05). The allele frequencies were similar in the PIAMA and KOALA cohorts (Figure 1A). Measures of LD were comparable between study populations. Strong LD was observed for rs3822357, rs3797054, rs14359 and IVS3-116, as D' approaches the value of 1. LD expressed as r^2 was remarkably lower (0.0-0.17), due to differences in allele frequencies between SNPs (Figure 1B).

Association with eczema in PIAMA and KOALA cohorts

In the PIAMA cohort (see Table 1 for characteristics), the del IVS3-116 variant was significantly associated with UKWP defined eczema at the age of 4 years, using the best-fitting dominant model (OR=1.90 (95% CI: 1.14-3.18)), and a tendency in a similar direction was observed at 1 year of age (OR=1.74 (0.81-3.72)) for this eczema definition. Furthermore IVS3-116 was border-line associated with increased risk of eczema, defined by questionnaire, in the PIAMA cohort (GEE analysis of 0-8 years; dominant model: (OR=1.33 (0.98-1.81)) (Table 2).

Figure 1: Protocadherin-1: Gene, mRNA expression, SNP position and LD-pattern. *PCDH1 SNPs are numbered 1 to 4 and corresponding minor allele frequencies are calculated per population (A). Linkage disequilibrium plots are displayed for D' and R-square values both for PIAMA and KOALA. D' is represented by black colour, meaning D' is >0.9. r^2=0 is white and r^2=1 is represented in black (B).*

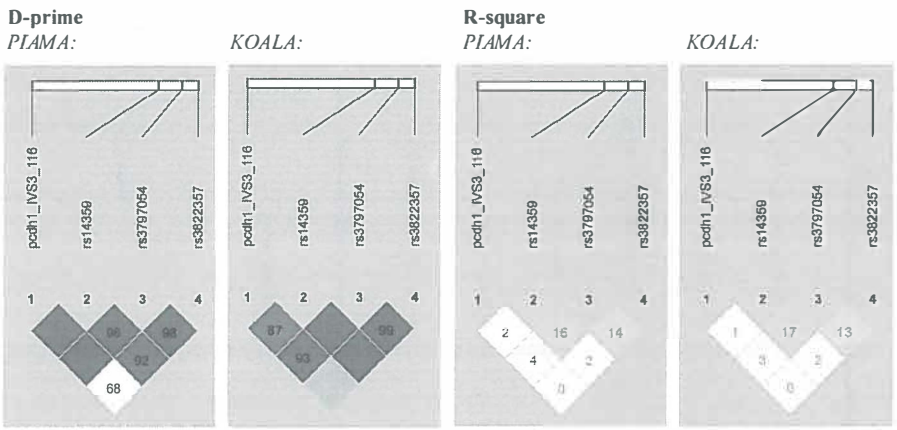
A



SNP	region	name	AC094107.3	rs-number	Allele 1	Allele 2	Frequency (allele 2) in cohorts	
							PIAMA	KOALA
1	exon 3	Ala514Thr	77474	rs3822357	G	A	0.08	0.07
2	exon 3	Ala750Ala	76764	rs3797054	T	C	0.36	0.36
3	exon 3	IVS3 +164	75751	rs14359	G	C	0.23	0.23
4	intron 3	IVS3 -116	70270	(submitted)	TTC Ins	TTC Del	0.06	0.07

gDNA = genomic DNA, SNP = Single Nucleotide Polymorphism, IVS = intervening sequence. Nucleotide sequence of IVS3_116 is as follows: gaatgtgctgtgctggggcctgggtctctt (-/ctt) ggttgctgttgaagcctgagtaggatggtg.

B



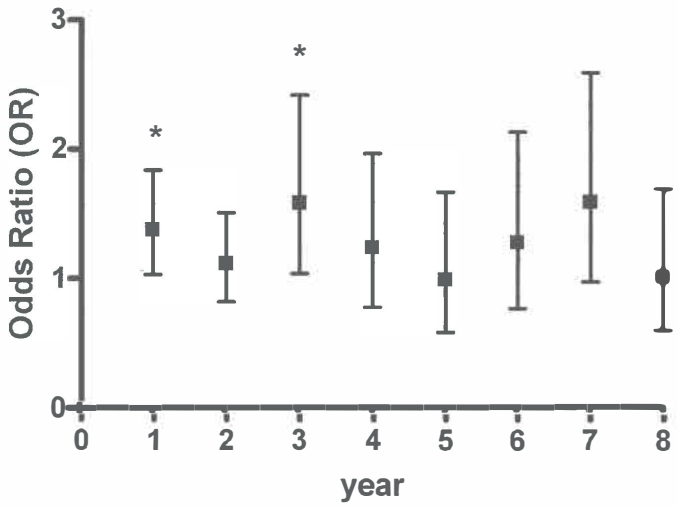
Rs3822357 (A-allele) was significantly associated with decreased risk of eczema, defined by questionnaire (GEE of 0-8 years, co-dominant model OR=0.19 (0.06-0.63)) in the PIAMA study. Numbers were too low to accurately determine association with UKWP criteria.

In the KOALA birth cohort (see Table 1 for characteristics) we also observed an association of IVS3-116 with increased risk of eczema. IVS3-116 del was significantly associated with ISAAC questionnaire defined eczema at 1 year (OR=1.44 (1.00-2.07),

dominant model), but not at later age and not with UKWP criteria (Table 2). The protective effect of rs3822357 (A-allele) was not identified in KOALA (Table 2). Other *PCDH1* SNPs were not significantly associated, neither with ISAAC questionnaire nor UKWP defined eczema, both in PIAMA and KOALA cohorts (data not shown).

Importantly, GEE analysis on pooled ISAAC questionnaire data of PIAMA and KOALA cohorts resulted in a significant association of IVS3-116 with eczema (OR=1.26 (1.01-1.58), dominant model) (Table 2 and Figure 2), but not of rs3822357 (OR=0.68 (0.23-2.03)). For IVS3-116 associations with eczema were specifically identified at age 1 and 3 years (OR=1.38 (1.03-1.84) and OR=1.59 (1.04-2.43)), and border-line at age 7 year (OR=1.60 (0.98-2.60), Figure 2). Adjustment for potential confounders (paternal atopy, sex, breastfeeding, intervention or study population) did not change any of the above mentioned associations.

Figure 2: Association of IVS3-116 with eczema in pooled PIAMA and KOALA cohorts. *Results of Generalized Estimating Equations are shown for IVS3-116. Odds ratios and 95% confidence intervals are calculated per year for pooled PIAMA and KOALA cohorts, using a dominant model (* = p<0.05).*



When we constructed haplotypes using the selected SNPs, the significant associated deletion variant IVS3-116 was only in a haplotype with wild type rs14359, rs3797054, and rs3822357 alleles, with similar allele frequencies as IVS3-116 alone (PIAMA 0.063; KOALA 0.070). Therefore the haplotype analysis did not result in different associations.

As *PCDH1* was previously associated with BHR and asthma, we investigated whether asthma status, at the same age as when the association of *PCDH1* with eczema was determined, had influence on the association of *PCDH1* with eczema. For the association of UKWP eczema at 4 year, we observed no confounding relationship of asthma phenotypes on the association of *PCDH1* with eczema. In addition, correction for doctor-diagnosed asthma had no influence on the association analyzed by GEE between *PCDH1* IVS3-116 and questionnaire defined eczema (OR=1.33 (0.98-1.81), p=0.07). Correction for asthma symptoms even showed a small increase in odds ratio (OR = 1.38 (0.99-1.92), p=0.058). Finally, in children with eczema, there was no significant association of *PCDH1* with BHR or asthma at later age (data not shown).

Table 2: Associations of PCDH1 SNPs with eczema in PIAMA and KOALA cohorts

Cohort	Eczema definition	Age (yrs)	SNP	# Total Subjects	Risk Allele	Outcome (OR (95%CI))	P-value
PIAMA	Questionnaire (GEE Analysis)	0-8	IVS3-116	961	TTC Del	(3) 1.33 (0.98-1.81)	0.070
	Questionnaire (GEE Analysis)	0-8	rs3822357	961	A	(1) 1.16 (0.84-1.61) (2) 0.19 (0.06-0.63)	0.36 0.0068
	UKWP	1	IVS3-116	392	TTC Del	(3) 1.74 (0.81-3.72)	NS
		4	IVS3-116	929	TTC Del	(3) 1.90 (1.14-3.18)	0.013
KOALA	Questionnaire	1	IVS3-116	1427	TTC Del	(3) 1.44 (1.00-2.07)	0.032
		2	IVS3-116	1483	TTC Del	(3) 0.91 (0.60-1.39)	NS
		6	IVS3-116	1283	TTC Del	(3) 1.10 (0.70-1.72)	NS
	Questionnaire	1	rs3822357	1438	A	(1) 0.75 (0.49-1.15) (2) 0.48 (0.06-3.84)	NS NS
		2	rs3822357	1496	A	(1) 0.90 (0.59-1.39) (2) 2.32 (0.60-9.05)	NS NS
		6	rs3822357	1290	A	(1) 0.63 (0.37-1.09) (2) 1.72 (0.35-8.33)	NS NS
	UKWP	2	IVS3-116 rs3822357			(3) 1.00 (0.52-1.92) (1) 0.72 (0.33-1.55) (2) 6.21 (0.86-44.73)	NS NS NS
Pooled	Questionnaire (GEE Analysis)	1-8 1-8	IVS3-116 rs3822357	2486 2486	TTC Del A	(3) 1.26 (1.01-1.58) (1) 1.03 (0.81-1.31) (2) 0.68 (0.23-2.03)	0.042 NS

Definition of models: (1) = Heterozygous minor allele compared to homozygous wild type, (2) = Homozygous minor allele compared to homozygous wild type, (3) = Dominant model (heterozygous and homozygous minor alleles compared to homozygous wild type). NS = not significant.

DISCUSSION

This study showed significant association between the *PCDH1* insertion deletion polymorphism IVS3-116 and increased risk of eczema, defined by objective measurements for eczema, the UK Working Party criteria. Moreover, in a pooled analysis of 2486 children of these birth cohorts, IVS3-116 was significantly associated with questionnaire defined eczema at age 0 to 8.

We observed association between the *PCDH1* polymorphism IVS3-116 and eczema in two independent Dutch birth cohorts. Importantly, the annual ISAAC core questions provided similar prevalence rates of eczema in these cohorts. We excluded children from non-Dutch origin and investigated a homogeneous Dutch population. Our studies were hypothesis driven, and we therefore did not correct for multiple testing but rather determined association of *PCDH1* polymorphisms in a second cohort. Furthermore, the same allele (del IVS3-116) was previously found to be associated with asthma and BHR (11), providing evidence that these diseases share a genetic background.

We detected several differences in the pattern of the association of *PCDH1* with eczema that related to an age specific effect, and the association of *PCDH1* with eczema defined through UKWP criteria. For KOALA, data on eczema is available in the first 2 years, and IVS3-116 associated with questionnaire eczema at age 1, but not at 2 or 6 years, in children not selected for maternal allergy. This may suggest that *PCDH1* is associated with an early onset of eczema. However, when analyzing PIAMA questionnaire data longitudinally with GEE, no change in risk was observed in time. In addition, when pooling the questionnaire data of both cohorts, a significant association was identified during the whole period in this larger sample size (Figure 2). Additionally, the association of IVS3-116 with eczema was not modified by paternal allergy.

The association of UKWP eczema with IVS3-116 was identified at the age of 4 years in a subset of the PIAMA cohort, while no association existed in KOALA at age 2 using this definition. One explanation for the difference in association of IVS3-116 with UKWP eczema can relate to the slightly different usage of UKWP-criteria. As in KOALA at the age of 2 years no data is available on asthma in children, the UKWP-criteria "History of asthma or hay fever" was not applied, while in PIAMA for the eczema assessment at the age of 4 years this criterion was included. This could have led to an enrichment of children in de PIAMA cohort with asthma or hay fever, and thereby influence the association. However, we detected no confounding relationship of asthma on the association of *PCDH1* with eczema. The detected

differences in the associations may relate to the onset or severity of eczema, or cohort specific gene-gene or gene-environmental interactions.

Although not strongly replicated, we do observe significant association in two independent cohorts. The significant associations were identified using 115 and 263 eczema subjects respectively. In order to investigate replication, we suggest analysing IVS3-116 in a larger sample size.

We did identify an association of the coding SNP rs3822357 (Ala514Thr) with eczema in PIAMA. Again, the same risk allele was observed for BHR in a family study from the UK and a birth cohort from the US (11). This finding was not significantly replicated in KOALA. This difference in association could not be explained by the difference in study design, as correction for potential confounders (paternal atopy, sex or breastfeeding) did not alter the association of rs3822357 with eczema in PIAMA. Given the low allele frequency of the risk allele, power was low to investigate replication and we therefore suggest that larger studies have to analyse this SNP in order to increase power to detect association.

Three *PCDH1* polymorphisms (rs3797054, IVS3-116, and rs3822357) have been previously reported to associate with BHR and asthma in Dutch, UK and US populations (11) and we now report that one of these polymorphisms (IVS3-116) to be associated with eczema. The functional effects of these gene variants are not known. Together with the published evidence on the adhesion function of PCDH1 (13) and its expression in skin keratinocytes (14), this points towards a role of this gene affecting epithelial integrity in BHR and asthma, a mechanism that also has been proposed for eczema (16, 28).

It is of interest to compare our results with data on filaggrin (*FLG*), a replicated gene for eczema, resulting in loss of epithelial integrity in the skin. Previously, we genotyped the PIAMA cohort for three *FLG* SNPs, and confirmed the relative high prevalence of the *FLG* null mutations in the north of Europe (29), which clearly differs for Mediterranean populations, were the frequency of these SNPs was low or not detected (30). These results point towards genetic heterogeneity of this disease. For *FLG*, we and others have observed an association with early age at onset of eczema (31-34). In addition, it has been reported that persons with eczema carrying *FLG* mutations are at increased risk to develop asthma. Albeit not that strong as for *FLG,* we do observe significant associations of relative rare *PCDH1* gene variants with eczema. We were not able to determine *FLG* and *PCDH1* gene-gene interactions due to low numbers of children carriers of both *FLG* and *PCDH1* deletions. Interestingly, for *PCDH1* we did not observe a progression towards asthma at age 6 to 8. Since the initial genetic association of *PCDH1* gene variants with asthma was identified in an

older age group, longer follow up may be needed to assess progression from eczema to asthma.

In conclusion, our study provides evidence for the association of *PCDH1* polymorphism IVS3-116 with eczema. Previously, IVS3-116 was shown to be associated with BHR. Our data show that *PCDH1* may have a pleiotropic effect on BHR and eczema in different populations.

ACKNOWLEDGMENTS

We would like to thank all participants from the PIAMA and KOALA birth cohorts. In addition we thank Judith Vonk for critically reading the manuscript. Henk Koning was supported by the European Commission as part of GABRIEL (A multidisciplinary study to identify the genetic and environmental causes of asthma in the European Community) contract number 018996 under the Integrated Program LSH-2004-1.2.5-1 and a grant from the University Medical Center Groningen. Funding PIAMA birth cohort: The Netherlands Organisation for Health Research and Development (ZonMW 2100.0090); the Netherlands Organisation for Scientific Research; the Netherlands Asthma Fund (EBO 3.2.03.48); the Netherlands Ministry of Spatial Planning, Housing, and the Environment; and the Netherlands Ministry of Health, Welfare and Sport. The KOALA birth cohort study was co-financed by: Netherlands Asthma Foundation, Netherlands Organisation for Health Research and Development (ZonMw), Royal Friesland Foods, Triodos Foundation, Foundation for the Advancement of Heilpedagogie, Phoenix Foundation, Raphaël Foundation, Iona Foundation, and Spinoza Award (2000, Prof. D.S. Postma). Genetic studies were supported by (ZonMw, The Netherlands Organisation for Health Research and Development, grant number 912-03-031 and 91656091 (ZON MW VENI grant to Dr Koppelman).

REFERENCES

1. Pearce N, Ait-Khaled N, Beasley R et al. Worldwide trends in the prevalence of asthma symptoms: phase III of the International Study of Asthma and Allergies in Childhood (ISAAC). Thorax 2007: 62: 758-66
2. Koppelman GH. Gene by environment interaction in asthma. Curr Allergy Asthma Rep 2006: 6: 103-11
3. Burgess JA, Dharmage SC, Byrnes GB et al. Childhood eczema and asthma incidence and persistence: a cohort study from childhood to middle age. J Allergy Clin Immunol 2008: 122: 280-5
4. Porsbjerg C, von Linstow ML, Ulrik CS et al. Risk factors for onset of asthma: a 12-year prospective follow-up study. Chest 2006: 129: 309-16
5. Van Beijsterveldt CE, Boomsma DI. Genetics of parentally reported asthma, eczema and rhinitis in 5-yr-old twins. Eur Respir J 2007: 29: 516-21

6. Beyer K, Nickel R, Freidhoff L et al. Association and linkage of atopic dermatitis with chromosome 13q12-14 and 5q31-33 markers. J Invest Dermatol 2000: 115: 906-8
7. Forrest S, Dunn K, Elliott K et al. Identifying genes predisposing to atopic eczema. J Allergy Clin Immunol 1999: 104: 1066-70
8. Doull IJ, Lawrence S, Watson M et al. Allelic association of gene markers on chromosomes 5q and 11q with atopy and bronchial hyperresponsiveness. Am J Respir Crit Care Med 1996: 153(4 Pt 1): 1280-4
9. Holloway JW, Lonjou C, Beghe B et al. Linkage analysis of the 5q31-33 candidate region for asthma in 240 UK families. Genes Immun 2001: 2: 20-4
10. Postma DS, Bleecker ER, Amelung PJ et al. Genetic susceptibility to asthma--bronchial hyperresponsiveness coinherited with a major gene for atopy. N Engl J Med 1995: 333: 894-900
11. Koppelman GH, Meyers DA, Howard TD et al. Identification of PCDH1 as a novel susceptibility gene for bronchial hyperresponsiveness. Am J Respir Crit Care Med 2009: 180: 929-35
12. Redies C, Vanhalst K, Roy F. delta-Protocadherins: unique structures and functions. Cell Mol Life Sci 2005: 62: 2840-52
13. Sano K, Tanihara H, Heimark RL et al. Protocadherins: a large family of cadherin-related molecules in central nervous system. EMBO J 1993: 12: 2249-56
14. Fitsialos G, Chassot AA, Turchi L et al. Transcriptional signature of epidermal keratinocytes subjected to in vitro scratch wounding reveals selective roles for ERK1/2, p38, and phosphatidylinositol 3-kinase signaling pathways. J Biol Chem 2007: 282: 15090-102
15. Nonnenmacher M, Salmon J, Jacob Y et al. Cottontail rabbit papillomavirus E8 protein is essential for wart formation and provides new insights into viral pathogenesis. J Virol 2006: 80: 4890-900
16. Sandilands A, Terron-Kwiatkowski A, Hull PR et al. Comprehensive analysis of the gene encoding filaggrin uncovers prevalent and rare mutations in ichthyosis vulgaris and atopic eczema. Nat Genet 2007: 39: 650-4
17. Brunekreef B, Smit J, de Jongste J et al. The prevention and incidence of asthma and mite allergy (PIAMA) birth cohort study: design and first results. Pediatr Allergy Immunol 2002: 13(Suppl 15): 55-60
18. Lakwijk N, Van Strien RT, Doekes G et al. Validation of a screening questionnaire for atopy with serum IgE tests in a population of pregnant Dutch women. Clin Exp Allergy 1998: 28: 454-8
19. Kummeling I, Thijs C, Penders J et al. Etiology of atopy in infancy: the KOALA Birth Cohort Study. Pediatr Allergy Immunol 2005: 16: 679-84
20. Williams HC, Burney PG, Hay RJ et al. The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis. I. Derivation of a minimum set of discriminators for atopic dermatitis. Br J Dermatol 1994: 131: 383-96
21. Williams HC, Burney PG, Strachan D et al. The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis. II. Observer variation of clinical diagnosis and signs of atopic dermatitis. Br J Dermatol 1994: 131: 397-405
22. Williams HC, Burney PG, Pembroke AC et al. The U.K. Working Party's Diagnostic Criteria for Atopic Dermatitis. III. Independent hospital validation. Br J Dermatol 1994: 131: 406-16
23. Kerkhof M, Koopman LP, Van Strien RT et al. Risk factors for atopic dermatitis in infants at high risk of allergy: the PIAMA study. Clin Exp Allergy 2003: 33: 1336-41
24. Snijders BEP, Stelma FF, Reijmerink NE, et al. CD14 polymorphisms in mother and infant, soluble CD14 in breast milk and atopy development in the infant (KOALA Study). Pediatr Allergy Immunol 2010: 21: 541-9
25. Kerkhof M, Postma DS, Brunekreef B, et al. Toll-like receptor 2 and 4 genes influence susceptibility to adverse effects of traffic-related air pollution on childhood asthma. Thorax 2010: 65: 690-7
26. Reijmerink NE, Kerkhof M, Koppelman GH et al. Smoke exposure interacts with ADAM33 polymorphisms in the development of lung function and hyperresponsiveness. Allergy 2009: 64: 898-904
27. Bottema RW, Reijmerink NE, Kerkhof M et al. Interleukin 13, CD14, pet and tobacco smoke influence atopy in three Dutch cohorts: the allergenic study. Eur Respir J 2008: 32: 593-602
28. Cookson W. The immunogenetics of asthma and eczema: a new focus on the epithelium. Nat Rev Immunol 2004: 4: 978-88
29. Chen H, Commen JEA, Haines RL et al. Wide spectrum of filaggrin-null mutations in atopic dermatitis highlights differences between Singaporean Chinese and European populations. Br J Dermatol 2011 March 24, Epub ahead of print.
30. Cascella R, Cuzzola VF, Lepre T et al. Full sequencing of the FLG gene in Italian patients with atopic eczema: Evidence of new mutations, but lack of an association. J Invest Dermatol 2011: 131 :982-984
31. Schuttelaar ML, Kerkhof M, Jonkman MF et al. Filaggrin mutations in the onset of eczema, sensitization, asthma, hay fever and the interaction with cat exposure. Allergy 2009: 64:1758-65
32. Stemmler S, Parwez Q, Petrasch-Parwez E et al. Two common loss-of-function mutations within the filaggrin gene predispose for early onset of atopic dermatitis. J Invest Dermatol 2007: 127: 722-4

33. Weidinger S, O'Sullivan M, Illig T et al. Filaggrin mutations, atopic eczema, hay fever, and asthma in children. J Allergy Clin Immunol 2008: 121: 1203-9
34. Bønnelykke K, Pipper CB, Tavendale R et al. Filaggrin gene variants and atopic diseases in early childhood assessed longitudinally from birth. Pediatr Allergy Immunol 2010: 21: 954–961.

Chapter 4

Characterization of Protocadherin-1 expression in Primary Bronchial Epithelial Cells: Association with epithelial cell differentiation

Henk Koning
Ian Sayers
Ceri E. Stewart
Debora de Jong
Nick H.T. ten Hacken
Dirkje S. Postma
Antoon J.M. van Oosterhout
Martijn C. Nawijn
Gerard H. Koppelman

FASEB J. 2012. Vol 26(1): p439-448

ABSTRACT

Protocadherin-1 (*PCDH1*) is a novel susceptibility gene for asthma that is expressed in airway epithelium. We aimed to characterize *PCDH1* mRNA transcripts and protein expression in primary bronchial epithelial cells, and to determine regulation of *PCDH1* during mucociliary differentiation. Total RNA and protein was isolated from human primary bronchial epithelial cells. *PCDH1* transcripts were characterized by Rapid Amplification of cDNA Ends in bronchial epithelial cells of four subjects. PCDH1 expression was quantified by qRT-PCR and western blotting in bronchial epithelial cells directly *ex vivo* and after air liquid interface (ALI) or submerged culture. We identified 5 novel exons on the 5'end and 1 exon on the 3'end of *PCDH1*. Novel transcripts showed major variation in expression of intracellular conserved motifs. Expression levels of *PCDH1* transcripts encoding exon 1-2 were 4-fold higher, and transcripts encoding exon 3-4 were 15-fold higher in freshly isolated bronchial epithelial cells than in submerged cultures. PCDH1 mRNA (3-8 fold) and protein levels (2-3 fold) were strongly upregulated during mucociliary differentiation of primary bronchial epithelial cells in ALI cultures. In conclusion, *PCDH1* transcripts display remarkable variability in expression of conserved intracellular signaling domains. Enhanced PCDH1 expression levels strongly correlate with differentiation of bronchial epithelial cells.

INTRODUCTION

Protocadherins are the largest subfamily of the cadherin superfamily of adhesion molecules (1). Protocadherins can be subdivided into clustered protocadherins (α-, β- and γ-protocadherins), flamingo (CELSR) cadherins, the large (Fat- and Dachsous-related) protocadherins, and non-clustered protocadherins (δ-protocadherins, PCDH12, PCDH20) (2;3). The δ-protocadherin family consists of δ1-protocadherins (PCDH1, PCDH7, PCDH9, PCDH11X/Y), and δ2-protocadherins (PCDH8, PCDH10, PCDH17, PCDH18, PCDH19) (4). δ-protocadherins are characterized by the presence of 7 (δ1) or 6 (δ2) extracellular cadherin repeats, and variable intracellular signaling domains with no similarity to classical cadherins (5). They are generally highly conserved, with a remarkable conservation of two intracellular signaling domains across evolution (6). The expression of clustered protocadherins is mainly restricted to the nervous system, but interestingly the non-clustered δ1-protocadherins are additionally expressed in a wide range of different tissues (7;8). δ1-protocadherins are implicated in several diseases, such as non small-cell lung cancer (PCDH7) (9), autism spectrum disorder (PCDH9) (10), and Alzheimer's disease (PCDH11) (11).

Recently, we identified Protocadherin-1 (*PCDH1*) as a novel susceptibility gene for asthma (12). PCDH1, as well as other members of the δ1-protocadherin sub-family, is characterized by the presence of three conserved motifs (CM1, -2 and 3) in the intracellular cytoplasmic tail (3). The CM1- and CM2 motifs are present in both δ1- and δ2-protocadherins, while the CM3-motif is characteristic for δ1-protocadherins only. CM3 has been shown to bind to the catalytic subunit of protein phosphatase 1 alpha (13), a protein implicated in lung morphogenesis (14). PCDH1 also contains a PDZ-domain Binding Site (PDZ-BS) at the C-terminal end of the cytoplasmic tail (15). The extracellular cadherin repeats of PCDH1 have been shown to display homotypic adhesion activity, but weaker than classical cadherins (16;17). Clearly, the function of PCDH1 is largely unknown, and hence its putative role in the pathogenesis of asthma.

PCDH1 mRNA is expressed in brain (6), skin (18), nose (19) and lung (12), as well as in a range of endothelial and epithelial cell lineages throughout mouse embryonic development (19). *PCDH1* mRNA expression is upregulated in a skin keratinocyte wounding model, suggesting a possible role of *PCDH1* in epithelial repair (18). We have shown expression of *PCDH1* in human peripheral blood mononuclear cells, fibroblasts, and primary

bronchial epithelial cells, and confirmed the presence of two *PCDH1* mRNA isoforms in the airway epithelium based on published annotated short 3 exon, and long 5 exon isoforms (12).

The expression of δ1-protocadherins in the nervous system is well characterized, but apart from their homotypic adhesion function, the exact function, expression and regulation of these genes is unknown. As δ1-protocadherins are highly conserved across evolution, analysis of the expression of PCDH1 in epithelial cells will contribute to the understanding of the function of the δ1-protocadherin family in general. Therefore, we aimed to characterize *PCDH1* mRNA and protein expression levels in freshly isolated and cultured primary bronchial epithelial cells. Moreover, we investigated if mucociliary differentiation of bronchial epithelial cells has an effect on *PCDH1* mRNA and protein expression levels.

MATERIAL AND METHODS

Cell culture

Primary bronchial epithelial cells (PBECs) were obtained by bronchial brushings of 19 asthma patients, using endoscopic cytology brushes (1.9mm, Cellebrity™, Boston-Scientific-International, France). All asthma patients had a doctor's diagnosis of asthma, showed reversibility and were hyperresponsive to histamine and/or adenosine 5'-monophosphate (AMP). Lung function measurements, histamine and AMP provocation tests, and atopy measurements have been described previously (20). All subjects gave written informed consent. Two bronchial brushings were collected from each subject. One brush was directly stored in RLT buffer (QIAGEN Benelux BV, Venlo, the Netherlands) for RNA extraction, while the second brush was collected in HBSS on ice for cell culture. Primary bronchial epithelial cells were cultured as described previously (21) for three passages before RNA and protein was extracted. The human bronchial epithelial cell-line 16HBE14o- (16HBE) was kindly provided by D.C. Gruenert (Department of Medicine, University of Vermont, and University of California, San Fransisco, USA) and cultured as described previously (22).

To investigate the potential role of PCDH1 in airway epithelial cell differentiation, a time-course series of air liquid interface cultured cells (ALIs) and submerged cultured cells was purchased (MucilAir™, Epithelix Sàrl, Geneva, Switzerland). These cells originated from bronchial airways of a healthy female donor (60 years). Bronchial epithelial cells were cultured under ALI and submerged conditions at Epithelix according to the following

protocol: bronchial airways were digested enzymatically for 2 days, and epithelial cells were isolated. 250,000 cells were seeded in each 24-wells Transwell insert, and cultured submerged for two days. Next, cells were cultured at air liquid interface or under submerged culture conditions for another 45 days (7 weeks). At time-points 1, 7, 21 and 45 days after start of ALI culture, cells were harvested both for RNA and protein in duplicate. Differentiation status was confirmed by FoxJ1 and ZO-1 protein expression levels, both markers of epithelial differentiation (23-25).

RNA and protein isolation

RNA was extracted using RNeasy Mini or Micro Elute kits according to the manufacturer's protocol (QIAGEN Benelux BV, Venlo, the Netherlands). Extensive in solution *DNase*I treatment (QIAGEN Benelux BV, Venlo, the Netherlands) was performed to remove gDNA traces. For isolation of total protein, T25 culture dishes and Transwell inserts were treated with Triton-X lysis buffer (1% Triton-X 100, 150mM NaCl, 5mM $MgCl_2$, 10mM HEPES), and 2x Leammli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125 M Tris HCl, pH 6.8), immediately boiled for 5 min and stored until further usage.

Rapid Amplification of cDNA Ends (RACE)

RACE-ready cDNA was generated using RNA isolated from four submerged cultured PBECs and 16HBE using RNA-ligase mediated RACE (GeneRacer-RLM-RACE Kit, Invitrogen, Carlsbad, USA), according to the manufacturer's protocol. Five- and 3' (nested) RACE reactions were performed using specific primer sets (Table 1), using PCR cycling conditions as described by manufacturer's protocol. The resulting PCR products were cloned into TOPO pCR2.1 plasmids using TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, USA). Twenty-five colonies (detection of transcripts with >4% abundance) were picked for each RACE reaction (5' and 3') for four patients and 16HBE. Plasmids were isolated using QIAGEN Miniprep kit (QIAGEN Benelux BV, Venlo, the Netherlands), and cloned PCR products were identified by nucleotide sequencing (M13-reverse or T7-forward primers, Baseclear, Leiden, The Netherlands).

For confirmation of the 5' PCDH1 RACE results, long range PCR reactions were performed from the newly discovered 5' exons towards either exon 2 UTR (primer 3656-Rev; corresponding to isoform 1), or towards exon 3 (primer 3327-Rev; corresponding to isoform 2), using Expand High Fidelity PCR System (Roche Diagnostics, Almere, the Netherlands)

(Table 1 for primer sequences). The 3' PCDH1 RACE results were confirmed by a PCR reaction spanning exon 3-5 (Table 1 for primer sequences). The DNA sequence of the PCR products was confirmed by nucleotide sequencing (Baseclear, Leiden, the Netherlands).

Determination of transcript quantities by quantitative Reverse Transcriptase (qRT)PCR

To determine the relative expression levels of individual PCDH1 transcripts, qRT-PCR assays were designed across exon-exon boundaries. qRT-PCR was performed using inventoried or in-house designed Taqman Assays (Applied Biosystems Europe BV, Nieuwekerk A/D IJssel, the Netherlands). qRT-PCRs were performed on the ABI7900HT cycler in 384-well format. For *PCDH1* two inventoried assays (assay exon 1-2, Hs00170174_m1; assay exon 3-4, Hs00260937_m1), and two On-Demand assays were purchased (assay exon 1B-1 and assay 1C-1; Applied Biosystems Europe BV, Nieuwekerk A/D IJssel, the Netherlands). In addition, pre-designed assays for four house-keeping genes were used: *GAPDH* (Hs99999905_m1), *β-actin* (Hs99999903_m1), *RPLPO* (Hs99999902_m1), and *β-2-microglobulin* (Hs99999907_m1). Assay exon 1F-1 was designed in house; FAM-labelled probe was ordered at Eurogentec, Liege, Belgium (Table 1 for primer sequences).

cDNA was produced using Omniscript™ Reverse Transcriptase (QIAGEN Benelux BV, Venlo, the Netherlands). A total of 1.75 µg of RNA was reverse transcribed into cDNA using Oligo-dT(12-18) primers in a 35 µl reaction volume, including RNase inhibitor, at 37°C for 1 hour. Each qRT-PCR reaction contained 50ng of cDNA, 250nM of probe, 900nM of forward and reverse primers, 5 µl TaqMan Universal PCR Master Mix (Applied Biosystems Europe BV, Nieuwekerk A/D IJssel, The Netherlands), in a final volume of 10 µl. All samples were measured in triplicate using recommended cycling conditions. Data was analyzed using SDS2.3 software by applying the ΔΔCt-method (Applied Biosystems User Bulletin 2). As four house-keeping genes were used, the best combination of house-keeping genes for normalization was determined by using the Normfinder applet (26). Efficiencies of all assays were determined by a 2-fold or 10-fold dilution series of pooled brush or cultured cells cDNA (Supplementary Table E1).

Table 1: GeneRacer and PCDH1 primers and combinations for 5'and 3' RACE reactions

Nr	Primer name	Primer sequence (5'-3')
RACE reaction primers		
1.	GeneRacer 5' primer	CGACTGGAGCACGAGGACACTGA
2.	GeneRacer 5' nested primer	GGACACTGACATGGACTGAAGGAGTA
3.	GeneRacer 3' primer	GCTGTCAACGATACGCTACGTAACG
4.	GeneRacer 3' nested primer	CGCTACGTAACGGCATGACAGTG
5.	5'RACE 684	GAGTGATGACTGGTGAGGCGAAG
6.	5'RACE nested 437	TAGCTTGTACAGGTGCCCCACAT
7.	3'RACE 2973	AAGTTCAACCTGATGAGCGATGC
8.	3'RACE nested 3213	TCCGACTACAGCTACCGCACCA
9.	3'RACE Ex3	CGCCGTCCAGCAAGTCATCCTCA
10.	3'RACE nested Ex3	CTGATGGCAGCATAGGAGAGATGGA
PCR exon 3-5 primers		
11.	Ex3-FW3b	GCCTGAGGATCACTATGAGCGCACCA
12.	Ex5-Rev2b	ACACGGGTCTTTCATTGACAGCTCAGCA
Long range PCR reaction primers		
13.	Ex1B-FW1	CTGGGGACAGGTGTTTGAGT
14.	Ex1C-FW1	CCAGATTCAGGCAGGAGAAGC
15.	Ex1F-FW3A	TACACCCAGCTGTGGATGAGATTCA
16.	Ex1D-FW1	TCGGCGTCCTCTGGGAG
17.	Ex1E-FW1	AGCAAGGAGGAAAAGAAGGAAAGAG
18.	3327-Rev	GGACGGCGTCTCAGACTCCT
19.	3656-Rev	TCAGTTATCCACAGGACCCCAG
qRT-PCR assay primers and probes		
20.	Ex1B-FW	ACTTCCCTTGCCTCGACTTC
21.	Ex1B-Rev	GGGAGGCCCCAGAATCAG
22.	Ex1B-Probe	FAM-ACAGGTGTTTGAGTAGGCC-NFQ
23.	Ex1C-FW	CGCCCCGCCACGTTA
24.	Ex1C-Rev	GGGAGGCCCCAGAATCAG
25.	Ex1C-Probe	FAM-TCGCGACATTATTATTCC-NFQ
26.	Ex1F-FW	TACACCCAGCTGTGGATGAGATTCA
27.	Ex1F-Rev	CCCAGAATCAGGAGGGCCTCTC
28.	Ex1F-Probe	FAM-TGTGCTGGGACTGACTGCTCTTGT-BHQ
RACE PCR reactions		
1A	GeneRacer 5' primer + 5'RACE 684	
1B	GeneRacer 5' nested primer + 5'RACE nested 437	
2A	GeneRacer 3' primer + 3'RACE 2973	
2B	GeneRacer 3' nested primer + 3'RACE nested 3213	
3A	GeneRacer 3' primer + 3'RACE Ex4	
3B	GeneRacer 3' nested primer + 3'RACE nested Ex3	

Detection of CpG-islands *in silico*

CpG-islands were detected using the Emboss CpGPlot program (http://www.ebi.ac.uk/Tools/emboss/cpgplot/), by submitting a sequence of 10kb upstream of exon 1, derived from genbank template AC094107.

Statistics

To test the differences between brushed PBEC and submerged cultured PBEC qRT-PCR results, a non-parametric paired Wilcoxon test was performed using SPSS 16.0 (IBM Netherlands BV, Nieuwegein, the Netherlands).

Detection of PCDH1 isoform 1, isoform 2, FoxJ1 and ZO-1 by Western-blotting

Both for cultured PBECs and ALI cultures, protein samples were separated on 8% acrylamide SDS-page gels (Bio-Rad Laboratories BV, Veenendaal, the Netherlands), or pre-cast NuView 4-20% gradient gels (Generon, Maidenhead, Berkshire, UK). Blotting procedures were performed as described previously (12), using affinity purified PCDH1 isoform specific polyclonal antibodies, FoxJ1 antibody (AF3619, R&D systems Europe, Abingdon, Oxon, United Kingdom), Zona Occludens (ZO)-1 antibody (33-9100, Zymed Laboratories, Invitrogen, Carlsbad, USA), and β-actin antibody (sc-47778, Santa-Cruz Biotechnology, Heidelberg, Germany). Polyclonal PCDH1 antibodies were generated from immunized rabbits against 15 amino acid peptide (QPFQLSTPQPLPHPYH, ep78) for isoform 1 and 15 amino acid peptide (SPSPPEDRNTKTAPV, ep76) for isoform 2 (Figure 1A). Antibodies were affinity column purified against immunizing peptides (Eurogentec, Liege, Belgium). Validation of antibodies is described in the Supplementary Data. Protein levels were quantified by densitometric analysis using Quantity One Software v4.6.2 (Bio-Rad Laboratories BV, Veenendaal, the Netherlands), relative to the β-actin loading control.

RESULTS

Identification of novel PCDH1 transcripts in primary bronchial epithelial cells

The *PCDH1* gene encodes two main isoforms through alternative splicing in brain (6): a short three exon isoform (Genbank ID: NM_002587) and a long five exon isoform (Genbank ID: NM_032420). The short isoform contains a specific sequence at exon 2, which is spliced out in the long isoform (Figure 1A). Rapid Amplification of cDNA Ends (RACE) was performed to identify the presence of annotated and possible novel *PCDH1* transcripts in

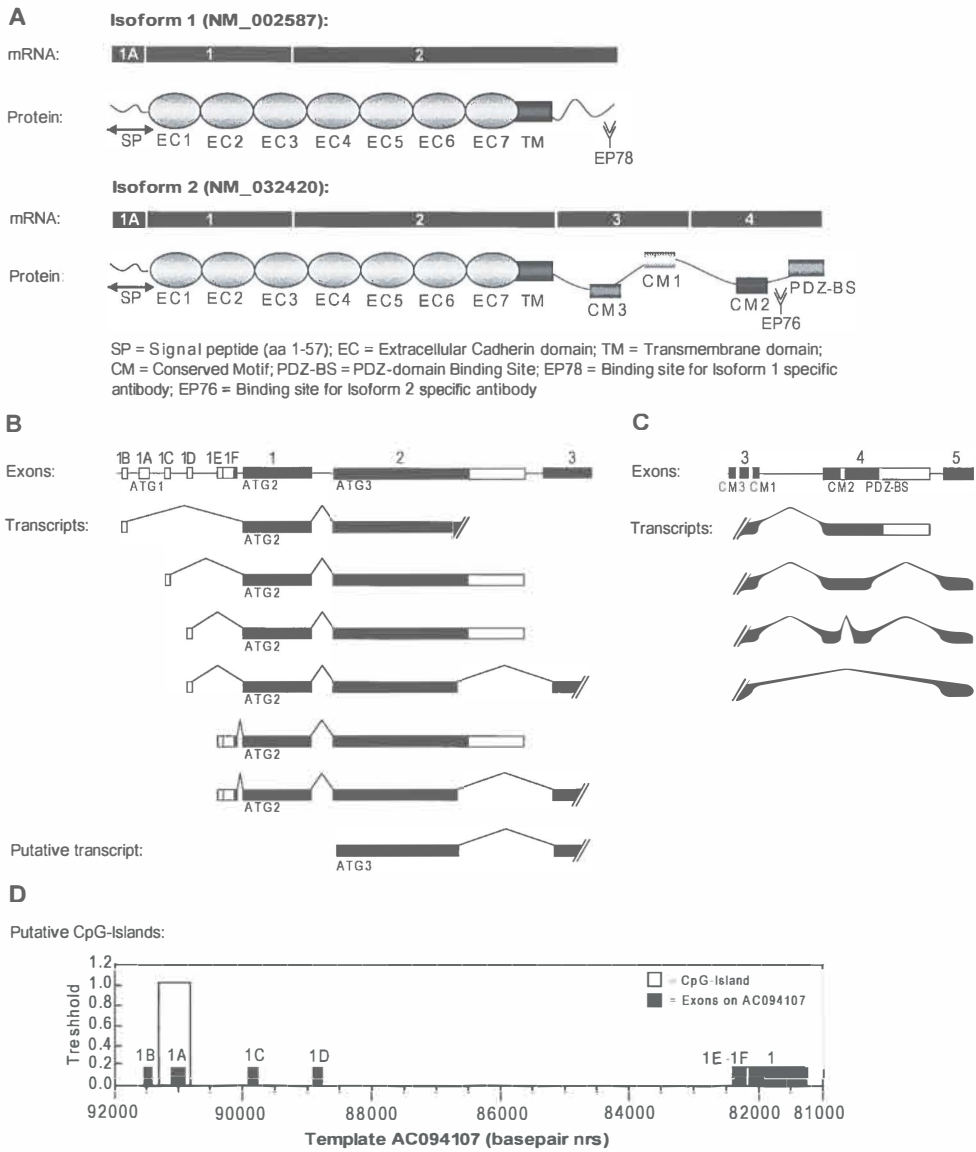
primary bronchial epithelial cells (PBECs) that had been cultured under submerged conditions for 3 passages. We revealed expression of five novel exons located 5' to annotated exon 1 of *PCDH1* (NM_002587) and one novel exon located 3' to annotated exon 4 of *PCDH1* (NM_032420) in cultured PBECs. In contrast, we did not detect expression of the annotated exon 1A previously observed in brain (6) (Figure 1B; Supplementary Figure E2). Expression of newly identified exons 1B – 1F was confirmed by sequence determination of transcript fragments PCR-amplified from cDNA samples of PBECs (Table 1 for primer sequences; Figure 1B for transcripts). We detected expression of novel 5' exons 1D, 1E and 1F in transcripts encoding both long and short *PCDH1* isoforms, whereas exons 1B and 1C were only detected in transcripts encoding the short isoform. All novel 5'exons were found in separate transcripts spliced directly to exon 1, with the exception of exon 1E, for which we also detected a transcript in which it was spliced to exon 1F and then to exon 1 (Figure 1B). In addition to novel 5' and 3' exons, we observed the presence of a gap within exon 4 or even full skipping of exon 4 in *PCDH1* transcripts of the long isoform (Figure 1C). Since exon 4 contains the highly conserved CM2 and PDZ-BS domains, these variants will affect the expression of these intracellular signaling domains (Figure 1A, C).

Culture of PBECs influences PCDH1 expression levels and detected isoforms

We designed quantitative RT-PCR assays spanning all novel exon – exon boundaries, and successfully validated these for transcripts containing the exon boundaries 1B-1, 1C-1 and 1F-1. As expression levels were found to be low for these transcripts, only presence or absence of these transcripts in cultured vs freshly isolated brush PBECs was determined. For submerged cultured PBECs, expression of transcripts encoding both exon 1C-1 (17/19 of subjects) and exon 1F-1 (18/19 of subjects) was detected, while transcripts encoding exon 1B-1 were not detected. In a preliminary study of 3 healthy subjects we also detected expression of transcripts encoding exon 1C-1 and 1F-1 in PBECs , but no expression of transcripts encoding exon 1B-1 (results not shown). In freshly isolated PBEC samples, expression of only the transcript encoding exon 1F-1 was detected in a subset of subjects (6/19).

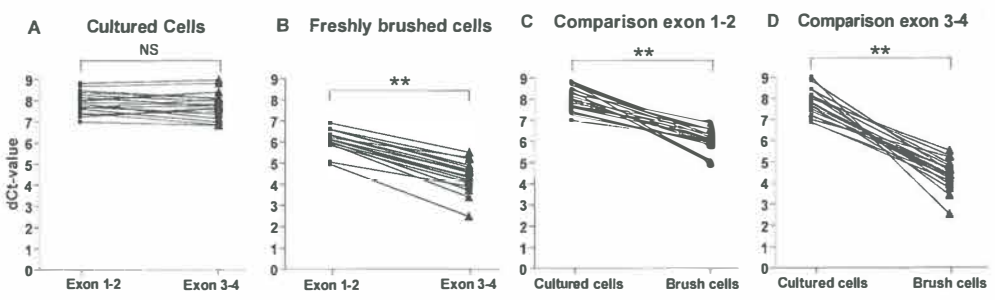
These results suggest that submerged culturing of cells induces alternative 5'exon usage in *PCDH1* transcripts. Epigenetic mechanisms can be an explanation for the low expression levels of these novel exons. Therefore we investigated by *in silico* analysis whether the exons were contained within a CpG-island. We detected a CpG-island of 565bp containing exon 1A, which was not detected by our RACE experiments, but no CpG-islands were detected around the other exons (Figure 1D).

Figure 1: Protocadherin-1 gene, RNA and protein structure.
(A) RNA and protein structure of PCDH1 isoform 1 and 2. Please note that exon numbering follows the annotation of mouse Pcdh1 by Van Roy and co-workers (6). Ep76 and Ep78 indicate the presence of the polypeptide sequence that was used to generate isoform specific antibodies. (B) Variation on 5'end of PCDH1 in primary bronchial epithelium of asthma patients. Novel exons were connected to isoform 1 or isoform 2 or both. Exons 1E and 1F exist both separately and connected to each other, both with isoform 1 and 2. (C) Variation on 3'end of PCDH1, which can result in alternative usage of conserved domains (CM2 and PDZ-BS). (D) A CpG-Island was detected surrounding exon 1A. Genbank accession numbers and prevalence of exons are provided in Supplementary Table E2.



In addition to exons 1B-1, 1C-1 and 1F-1, we quantified expression of transcripts containing exon 1-2 and exons 3-4 boundaries in PBECs. According to the annotated gene structure of *PCDH1*, qRT-PCR assay for exon 1-2 can detect both short and long *PCDH1* isoforms, while the exon 3-4 assay is specific for the long isoform. *PCDH1* expression levels in submerged cultured PBECs and freshly isolated brush samples were quantified relative to the average of three house-keeping genes (*β-actin*, *GAPDH*, *RPLP0*) using pre-validated qRT-PCR assays. We detected similar expression levels of *PCDH1* transcripts with the exon 1-2 and exon 3-4 assays in cultured PBECs, indicating that almost all of *PCDH1* transcripts encode PCDH1 isoform 2 (Figure 2A). Surprisingly, and in strong contrast to cultured PBECs, higher expression levels of *PCDH1* transcripts were detected with the exon 3-4 assay compared to the exon 1-2 assay in freshly isolated PBECs of all 19 subjects (Figure 2B). *PCDH1* expression levels in PBECs from 3 healthy controls displayed a very similar level and regulation between culture conditions as those in PBECs of asthma patients (results not shown). The expression difference between exon 1-2 and 3-4 indicates that a *PCDH1* mRNA transcript exists in freshly isolated PBECs, that contains exon 3 and 4, but not exon 1 and/or 2, encoding the extracellular and transmembrane domains. This novel *PCDH1* transcript has not been reported before, and was not identified by our RACE approach since we used primers located in exon 1 to characterize the 5` sequences of the *PCDH1* transcripts, and performed our analysis in submerged cultured PBECs that may not contain this novel transcript.

Figure 2: Expression of exon 1-2 and 3-4 in submerged cultured cells and freshly brushed cells.
*(A, B) Quantification of transcripts encoding PCDH1 exon 1-2 and exon 3-4 in submerged cultured and freshly brushed cells. (C, D) Comparision of exon 1-2 and exon 3-4 expression between submerged cultured and brush cells. dCt = delta-Ct values; NS=non-significant; ** = p<0.0001.*



Therefore, we performed additional 5' RACE experiments from exon 3 in differentiated air liquid interface cultured PBECs to identify a putative novel transcript encoding exons 3-4 but lacking exons 1 and/or 2. Indeed we identified a *PCDH1* transcript that does not encode exon 1, and presumably starts translation from within exon 2, where an in-frame start codon is present in the context of a strong KOZAK sequence (TCAGTG*ATG*GAG, ATG3, Figure 1A), allowing the expression of a PCDH1 protein encoding the extracellular EC6 and EC7 domains, the transmembrane and intracellular domains, but missing the first five extracellular cadherin repeats. In addition to this novel *PCDH1* transcript, we also observed significantly lower *PCDH1* mRNA expression levels in submerged cultured cells compared to freshly isolated PBECs ($p<0.0001$), both with the exon 1-2 assay (Figure 2C) and the exon 3-4 assay (Figure 2D). Specifically, exon 1-2 expression levels were 4-fold higher in freshly isolated PBECs, while exon 3-4 expression levels were 15-fold higher in freshly isolated than in cultured PBECs. These data show that higher *PCDH1* mRNA expression levels are observed in freshly *ex vivo* isolated brush cells compared to submerged cultured PBECs.

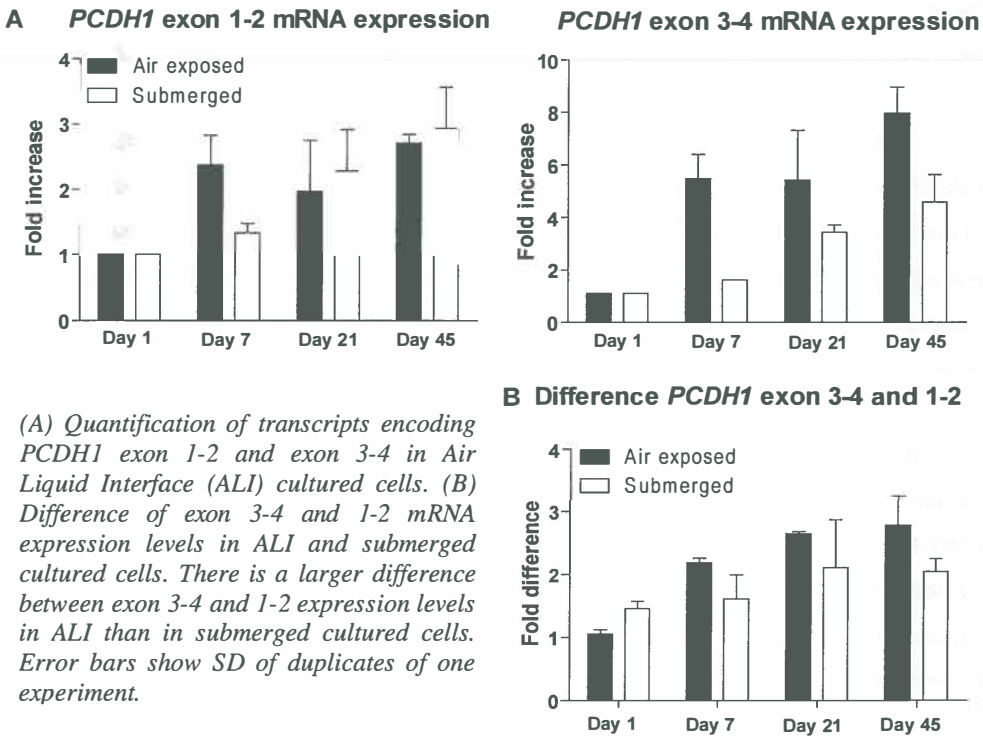
We found evidence for three PCDH1 transcripts: (i) the short annotated isoform encoding exon 1 and exon 2 and displaying alternative upstream exon usage, (ii) the long annotated isoform encoding exons 1, 2, 3 and 4 and using alternative upstream exons and (iii) a novel *PCDH1* transcript encoding exon 3-4 but not exon 1 and only part of the coding sequences of exon 2, identified by RACE in differentiated PBECs. We next aimed to identify the protein products encoded by these different transcripts. To this end, we first determined which PCDH1 protein products were expressed in submerged cultured PBECs and 16HBE using novel antibodies that were raised to peptides specific to the cytoplasmic tail of the two annotated PCDH1 isoforms (Figure 1A, Supplementary Figure E1 for validation). The antibody specific to the exon-2 encoded intracellular sequences (annotated isoform 1) detected the full-length protein only, whereas the antibody specific to the exon-4 encoded intracellular sequences (annotated isoform 2) detected both the full-length protein and a shorter protein isoform of around 50 kD (Figure E1A). The size of the latter 50 kD protein is consistent with a PCDH1 protein product encoded by the newly identified transcript. In cultured PBECs, we detected hardly any expression of the annotated PCDH1 protein isoform 1, in agreement with our qRT-PCR data (Supplementary Figure E2A and B). The antibody specific to annotated isoform 2 detected two PCDH1 protein products: one corresponding to the full-length protein (170 kD), that was detected in cultured PBECs and 16HBE, and the

short protein product of approximately 50 kD in 16HBE, but not in cultured PBECs, which is consistent with our qRT-PCR data (Figure E2B).

PCDH1 expression levels increase during air liquid interface culture conditions

We observed a strong reduction of *PCDH1* mRNA expression levels during the 3 passages of submerged culture used to expand the PBECs, compared to freshly brushed cells of the same subject (Figure 2). This expression difference between cultured and freshly brushed PBECs was not restricted to PBECs of asthma patients, as we observed the same expression pattern after preliminary analysis of *PCDH1* expression levels in PBECs of healthy subjects (results not shown).

Figure 3: PCDH1 mRNA expression in air liquid interface cultures of epithelial cells over time.



(A) Quantification of transcripts encoding PCDH1 exon 1-2 and exon 3-4 in Air Liquid Interface (ALI) cultured cells. (B) Difference of exon 3-4 and 1-2 mRNA expression levels in ALI and submerged cultured cells. There is a larger difference between exon 3-4 and 1-2 expression levels in ALI than in submerged cultured cells. Error bars show SD of duplicates of one experiment.

We hypothesized that the loss of mucociliary differentiation of PBECs due to submerged culture conditions (27) reduced *PCDH1* expression levels in PBECs. We therefore compared *PCDH1* mRNA expression levels between PBECs of healthy subjects grown under submerged and those grown under air liquid interface (ALI) culture conditions, as ALI

cultures have been described to induce mucociliary differentiation in primary bronchial epithelial cells (35) (Figure 3).

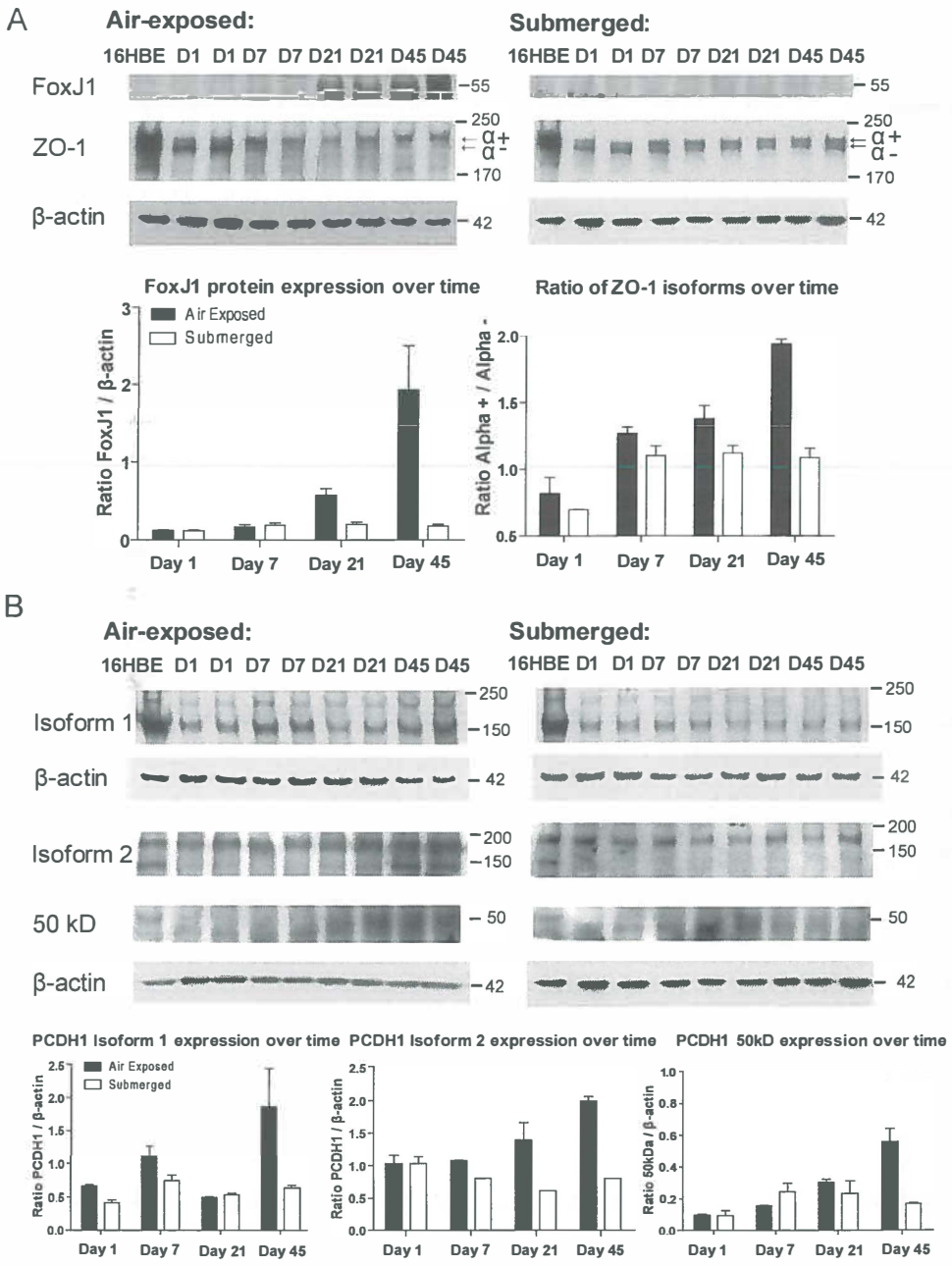
We confirmed the kinetics of this differentiation in our ALI cultures using the ciliated-cell specific protein FoxJ1 (23) and the ratio of the isoforms of ZO-1 associated with differentiation (24, 25) (Figure 4A). We observed a strong increase (3-8 fold) in *PCDH1* mRNA expression levels with both exon 1-2 and exon 3-4 assays after one week of air liquid interface culture, compared to the first day of ALI culture. *PCDH1* mRNA expression levels gradually increased to reach similar levels at week 7 in ALI and submerged cultures when detected with the exon 1-2 assay, but remained much higher in ALI than submerged cultures at all times when detected with exon 3-4 assay (Figure 3A).

As seen before (Figure 2), equal expression levels of transcripts encoding exon 1-2 and transcripts encoding exon 3-4 were observed at day 1, when cells were transferred to ALI culture conditions from submerged cultures. In contrast, at day 45 of ALI culture, a 2.8-fold higher expression of *PCDH1* transcripts was detected with the exon 3-4 assay compared to the exon 1-2 assay. For submerged cultures this difference was less pronounced (Figure 3B).

To determine whether the upregulation of *PCDH1* mRNA levels in differentiated ALI cultures is also observed at the protein level, we analyzed protein levels of PCDH1 isoforms in the 7-week PBEC cultures, maintained either under ALI or submerged culture conditions. Both PCDH1 full-length protein isoforms (corresponding to the extracellular domains, the transmembrane domain and the intracellular domains for both annotated isoforms) were strongly (2-3 fold) increased after 7 weeks of ALI culture compared to submerged culture conditions (Figure 4B). In addition, we detected a 50kD protein product, previously detected in 16HBE (Figure E2B), at the latest time points (day 21 and day 45) of air liquid interface culture, with higher expression in ALI cultured PBECs compared to submerged cultured PBECs (Figure 4B). These data indicate that PCDH1 protein expression levels during PBEC culture by and large follow the kinetics of the qRT-PCR data, with elevated PCDH1 expression under ALI culture conditions, both for the long PCDH1 isoforms and the 50 kD protein product.

Figure 4: PCDH1 isoform 1 and isoform 2 protein expression in air liquid interface cultures of epithelial cells over time.
(A) Mucociliary differentiation is indicated by FOXJ1 and ZO-1 Alpha + expression, which are both markedly upregulated in ALI culures, but not in submerged cultures. (B) An increase

of PCDH1 isoform 1 and 2 expression is observed, with the highest expression at D45 (day 45). Submerged cultures show no clear increase in PCDH1 expression. Graphs show densitometric quantification of PCDH1 bands on western blots, relative to β-actin. A representative example of 3 independent experiments with comparable results is shown. Error bars respresent SD of duplicates.



DISCUSSION

We recently identified protocadherin–1 (*PCDH1*) as a novel gene for asthma that is expressed in airway epithelial cells. PCDH1 is a member of the δ1-protocadherins, which encode transmembrane proteins with 7 extracellular cadherin repeats and in some isoforms intracellular conserved protein-protein interaction domains that are involved in signal transduction. δ1-protocadherins have been shown to engage in homotypic interactions, but otherwise the functional role of these proteins is unknown. To explore a possible functional role for PCDH1 in bronchial epithelial cells, we first characterized the *PCDH1* transcripts present in PBECs and the regulation of *PCDH1* isoform expression during PBEC mucociliary differentiation in the ALI culture model.

Identification of novel PCDH1 mRNA isoforms

The starting points of our analysis were the annotated *PCDH1* mRNA isoforms, originally identified in brain tissue: a 3 exon (isoform 1, Genbank ID NM_002587) and a 5 exon isoform (isoform 2, Genbank ID NM_032420), Figure 1A). Both isoforms share an extracellular and a transmembrane domain, but differ in their intracellular domains: isoform 1 carries a short unique cytoplasmic region encoded by exon 2, whereas isoform 2 has a long cytoplasmic region encoding the conserved signaling domains encoded by exons 3 and 4 (6). In our RACE experiments on cultured PBECs, we identified five novel *PCDH1* 5'exons that are mainly expressed in undifferentiated cells during submerged cell culture (Figure 2). By long-range PCR exons 1B and 1C were shown to be present only in transcripts encoding the short PCDH1 isoform, while exons 1D, 1E and 1F were present in transcripts encoding both PCDH1 isoforms. The alternative usage of 5' exons between the transcripts terminating at the exon 2 UTR (the annotated short isoform) and those terminating at the exon 4 UTR (the annotated long isoform), indicates that promoter usage might be different between these two main isoforms. Remarkably, we did not detect the annotated exon 1A. PCR amplification of exon 1A may, however have been hampered by the very high GC content (81.5 %) of this exon, precluding identification by RACE as well as by RT-PCR. In fact, exon 1A is located within a CpG island, indicating that epigenetic mechanisms may contribute to regulation of *PCDH1* gene expression (Figure 1D). The annotated *PCDH1* isoforms contain two in-frame start codons, one in exon 1A (ATG1), and one in exon 1 (ATG2). In addition, we identified a putative additional in-frame start codon in exon 2 (ATG3) in the context of a strong KOZAK sequence. Of the five novel 5' exons, only exon 1F contains an in-frame ATG sequence, but

no conserved Kozak sequence, hence it is a less likely translation initiation site. The lack of conserved translational start sites and relatively low expression of the novel 5' exons suggest that these 5'exons may contribute to mRNA translation regulation as part of the 5'UTR sequences. Vanhalst and co-workers suggested that translation initiation of *PCDH1* employs the first start-codon of exon 1 (ATG2), since the corresponding Kozak sequence of ATG2 was best conserved between species (6).

We investigated the expression levels of *PCDH1* in 3 healthy controls. *PCDH1* exon 1-2 and 3-4 expression levels were by and large similar between healthy controls and asthmatic subjects, but a thorough comparison between healthy and asthma will be the focus of a future study. Furthermore we investigated whether *PCDH1* exon 1-2 and 3-4 expression levels were influenced by three *PCDH1* gene-variants (IVS3-116, rs14359, and rs3797054), previously associated with bronchial hyperresponsiveness and asthma (12). With expression levels of 19 subjects we unfortunately were underpowered for this analysis. One of our most remarkable findings was the detection of higher *PCDH1* mRNA expression levels with an exon 3-4 specific assay than an exon 1-2 specific assay, both in freshly brushed epithelial cells and at later time-points in the ALI cultured PBECs, but not in submerged cultured PBECs. These data were in contrast to our expectations based on the published annotated isoforms of *PCDH1*. We expected *PCDH1* expression detected by the exon 1-2 specific assay to reflect expression of both isoforms, thereby constituting the total *PCDH1* mRNA pool, whereas *PCDH1* expression detected by the exon 3-4 specific assay was expected to reflect the level of the long 5-exon isoform only. Based on our results we here hypothesize the presence of a novel putative *PCDH1* transcript lacking exon 1 and/or 2. Importantly, the expression difference between exon 1-2 and exon 3-4 was not due to lower efficiency of the exon 1-2 qRT-PCR assay (Supplementary Table E1). For this putative variant, translation may start from within exon 2, where an in frame start-codon is present within a Kozak-sequence (ATG3). In RACE experiments, we confirmed the presence of a transcript variant carrying ATG3, but not ATG2 or ATG1 (Figure 1A). Translational initiation from the putative ATG3 start codon located in exon 2 would result in a truncated protein product, lacking 5 of the extracellular domains, but retaining the conserved intracellular signaling domains. As adhesion typically forms by engagement of the EC1 domain, this isoform would not be able to participate in cell-cell adhesion, but could still participate in signal transduction. A truncated variant was previously described for PCDH15, where PCDH15 isoform B has a deletion of 10 out of 11 cadherin domains, but still retains the transmembrane domain and

intracellular domains (28). Interestingly, we did identify a PCDH1 protein product of 50 kD using the antibody directed against the intracellular part of isoform 2. This 50kD product could be the protein product of the putative novel isoform, although we can not exclude the possibility that this product is the result of a full-length isoform modified by posttranslational cleavage or shedding. Such a shedding process was previously identified for the clustered gamma-protocadherins and the non-clustered Protocadherin-12 (29; 30), but is yet unknown for δ1-protocadherins. Future studies such as proteomic analysis will be performed for the characterization of this novel PCDH1 protein.

δ1-protocadherins are characterized by 3 conserved motifs in their intracellular tail (CM1-CM3), of which the function of CM1 and CM2 is unknown. At the 3'end of *PCDH1* mRNA we observed substantial variability in the transcription of exon 4: we identified transcripts with an intra-exon gap (in-frame deletion of 96 basepairs) as well as transcripts with exon skipping of exon 4. This gap in exon 4 has also been described to be present in mouse *Pcdh1* (Genbank ID: AY861418), but has no consequence for the expression of conserved domains (6). In contrast, deletion of the entire exon 4 would result in the translation of a PCDH1 protein lacking CM2, and a putative PDZ-domain binding site. The CM2 motif is strongly conserved during metazoan evolution, and therefore considered as an important motif for downstream functions of many protocadherins (31). However, function and putative binding partners for CM2 have not been identified to date. PDZ-containing proteins can provide linkage to the cytoskeleton, or initiate signaling functions either directly or indirectly by acting as scaffolds for large protein complexes. Deletion of the PDZ-BS might therefore potentially influence coupling of PCDH1 to the cytoskeleton or downstream signaling functions (15;32). Besides PCDH1, the δ1-protocadherin PCDH11 has also been shown to be subject to complex splicing patterns, resulting in several transcripts with alternative expression of conserved motifs (33). Thus, δ1-protocadherins seem to have multiple protein isoforms with different use of the three intracellular CM-domains, allowing the specific protein isoforms to selectively address certain, as of yet unknown, signalling pathways depending on the presence or absence of the different CM domains. Future research will be needed to identify the relevant signalling pathways regulated by the δ1-protocadherins, as well as the regulation of splicing events leading to inclusion or exclusion of specific CM domains. We here thus describe evidence for an intriguing diversity of *PCDH1* transcripts with strongly variable 5'and 3'exon usage, likely affecting protein function, and additionally for a novel transcript isoform encoding the intracellular and transmembrane domains, but only containing two of the extracellular cadherin repeats. Given the strong conservation between

δ1-protocadherin family members, these data are likely to be of relevance to the other family members as well.

Interestingly, we observed higher *PCDH1* mRNA expression levels in freshly isolated cells than in PBECs of the same subject cultured for 3 passages under submerged conditions, both with the exon 1-2 and exon 3-4 specific assays. The cells obtained by bronchial brushing are about 85-90% epithelial cells (34;35), and we consider these brushed PBECs to be terminally differentiated. In contrast, submerged tissue culture conditions are likely to render undifferentiated cells, especially after multiple passages (27;36). We therefore hypothesized that *PCDH1* expression levels are regulated by the differentiation status of bronchial epithelial cells. In agreement with this, we observed increased *PCDH1* mRNA and protein expression levels in PBECs cultured under ALI conditions, compared to submerged cultured cells. Since ALI cultures have been shown to induce terminal mucociliary differentiation of PBECs (35;36) and we also observed the same differences in PCDH1 expression levels between freshly isolated (terminally differentiated) PBECs from bronchial brushes and cultured (relatively undifferentiated) cells, we conclude that high PCDH1 expression levels are associated with terminal differentiation of bronchial epithelial cells.

We are the first to show an increase of Protocadherin-1 expression at the mRNA and protein level over time in differentiated ALI cultures. Together with a proposed role of PCDH1 in cell-cell adhesion, and its identification as an asthma susceptibility gene, this suggests that there may be a role for PCDH1 in epithelial differentiation. Previously Protocadherin of the Liver, Kidney and Colon (PCDH-LKC/CDHR2) was shown to be regulated during Madin-Darby Canine Kidney (MDCK) epithelial cell differentiation (38).

Furthermore, RNA expression of Protocadherin-7, a closely related δ1-protocadherin gene, was shown to be upregulated during bronchial epithelial cell differentiation in a microarray experiment (39). Therefore, the increase of protocadherin expression levels during epithelial differentiation might be a common regulatory mechanism for the non-clustered δ1-protocadherins. Epithelial differentiation encompasses several processes, including upregulation of adhesion molecules, establishment of apical/basolateral cell polarity and planar cell polarity, and development of cilia (40). Asthma is characterized by a decreased epithelial barrier function (41) and abnormal repair of epithelial cells (42-44). One aspect of epithelial repair is the re-growth of columnar epithelial cells or differentiation and proliferation of basal epithelial cells (45). We propose that dysregulation of PCDH1

expression results in slower or incomplete differentiation of the epithelial layer, thereby contributing to an altered response to injury and a weaker epithelial barrier function.

ACKNOWLEDGMENTS

We would like to thank Dr. C. van Kooten (Leiden University Hospital, the Netherlands) for providing mouse L-cell fibroblasts, and D.C. Gruenert (Department of Medicine, University of Vermont, and University of California, San Fransisco, CA, USA) for providing the bronchial epithelial cell-line 16HBE14o-. Furthermore we would like to thank all subjects who contributed to this study and J. Noordhoek for technical assistance. This study was funded by Jan Kornelis de Cock stichting, GABRIEL: a European Commission FP6 grant, Stichting Astma bestrijding, Netherlands Asthma Foundation grant 3.2.09.055, and ZonMW Veni Koppelman (916.56.091).

REFERENCES

1. Frank, M., Kemler, R. (2002) Protocadherins. Curr.Opin.Cell Biol. 14, 557-562
2. Kim, S. Y., Yasuda, S., Tanaka, H., Yamagata, K., Kim, H. (2011) Non-clustered protocadherin. Cell Adh.Migr. 5, 97-105
3. Redies, C., Vanhalst, K., Roy, F. (2005) delta-Protocadherins: unique structures and functions. Cell Mol.Life Sci. 62, 2840-2852
4. Hulpiau, P., Van Roy, F. (2009) Molecular evolution of the cadherin superfamily. Int J Biochem Cell Biol. 41, 349-69
5. Wu, Q., Maniatis, T. (1999) A striking organization of a large family of human neural cadherin-like cell adhesion genes. Cell 97, 779-790
6. Vanhalst, K., Kools, P., Staes, K., van Roy, F., Redies, C. (2005) delta-Protocadherins: a gene family expressed differentially in the mouse brain. Cell Mol.Life Sci. 62, 1247-1259
7. Strehl, S., Glatt, K., Liu, Q. M., Glatt, H., Lalande, M. (1998) Characterization of two novel protocadherins (PCDH8 and PCDH9) localized on human chromosome 13 and mouse chromosome 14. Genomics 53, 81-89
8. Yoshida, K., Yoshitomo-Nakagawa, K., Seki, N., Sasaki, M., Sugano, S. (1998) Cloning, expression analysis, and chromosomal localization of BH-protocadherin (PCDH7), a novel member of the cadherin superfamily. Genomics 49, 458-461
9. Huang, Y. T., Heist, R. S., Chirieac, L. R., Lin, X., Skaug, V., Zienolddiny, S., et al (2009) Genome-wide analysis of survival in early-stage non-small-cell lung cancer. J.Clin.Oncol. 27, 2660-2667
10. Marshall, C. R., Noor, A., Vincent, J. B., Lionel, A. C., Feuk, L., Skaug, J., Shago, M., Moessner, et al. (2008) Structural variation of chromosomes in autism spectrum disorder. Am.J.Hum.Genet. 82, 477-488
11. Carrasquillo, M. M., Zou, F., Pankratz, V. S., Wilcox, S. L., Ma, L., Walker, L. P., Younkin, S. G., et al (2009) Genetic variation in PCDH11X is associated with susceptibility to late-onset Alzheimer's disease. Nat.Genet. 41, 192-198
12. Koppelman, G. H., Meyers, D. A., Howard, T. D., Zheng, S. L., Hawkins, G. A., Ampleford, E. J., et al (2009) Identification of PCDH1 as a novel susceptibility gene for bronchial hyperresponsiveness. Am.J.Respir.Crit Care Med. 180, 929-935
13. Yoshida, K., Watanabe, M., Kato, H., Dutta, A., Sugano, S. (1999) BH-protocadherin-c, a member of the cadherin superfamily, interacts with protein phosphatase 1 alpha through its intracellular domain. FEBS Lett. 460, 93-98
14. Hormi-Carver, K. K., Shi, W., Liu, C. W., Berndt, N. (2004) Protein phosphatase 1alpha is required for murine lung growth and morphogenesis. Dev.Dyn. 229, 791-801

15. Demontis, F., Habermann, B., Dahmann, C. (2006) PDZ-domain-binding sites are common among cadherins. Dev.Genes Evol. 216, 737-741
16. Morishita, H., Yagi, T. (2007) Protocadherin family: diversity, structure, and function. Curr.Opin.Cell Biol. 19, 584-592
17. Sano, K., Tanihara, H., Heimark, R. L., Obata, S., Davidson, M., St John, T., Taketani, S., Suzuki, S. (1993) Protocadherins: a large family of cadherin-related molecules in central nervous system. EMBO J. 12, 2249-2256
18. Fitsialos, G., Chassot, A. A., Turchi, L., Dayem, M. A., LeBrigand, K., Moreilhon, C., et al (2007) Transcriptional signature of epidermal keratinocytes subjected to in vitro scratch wounding reveals selective roles for ERK1/2, p38, and phosphatidylinositol 3-kinase signaling pathways. J.Biol.Chem. 282, 15090-15102
19. Redies, C., Heyder, J., Kohoutek, T., Staes, K., van Roy, F. (2008) Expression of protocadherin-1 (Pcdh1) during mouse development. Dev.Dyn. 237, 2496-2505
20. Broekema, M., ten Hacken, N. H., Volbeda, F., Lodewijk, M. E., Hylkema, M. N., Postma, D. S., Timens, W. (2009) Airway epithelial changes in smokers but not in ex-smokers with asthma. Am.J.Respir.Crit Care Med. 180, 1170-1178
21. Borger, P., Kauffman, H. F., Scholma, J., Timmerman, J. A., Koeter, G. H. (2002) Human allogeneic CD2+ lymphocytes activate airway-derived epithelial cells to produce interleukin-6 and interleukin-8. Possible role for the epithelium in chronic allograft rejection. J.Heart Lung Transplant. 21, 567-575
22. Heijink, I. H., Brandenburg, S. M., Noordhoek, J. A., Postma, D. S., Slebos, D. J., van Oosterhout, A. J. (2010) Characterisation of cell adhesion in airway epithelial cell types using electric cell-substrate impedance sensing. Eur.Respir.J. 35, 894-903
23. You, Y., Huang, T., Richer, E. J., Schmidt, J. E., Zabner, J., Borok, Z., Brody, S. L. (2004) Role of f-box factor foxj1 in differentiation of ciliated airway epithelial cells. Am.J.Physiol Lung Cell Mol.Physiol 286, L650-L657
24. Ciana, A., Meier, K., Daum, N., Gerbes, S., Veith, M., Lehr, C. M., Minetti, G. (2010) A dynamic ratio of the alpha+ and alpha- isoforms of the tight junction protein ZO-1 is characteristic of Caco-2 cells and correlates with their degree of differentiation. Cell Biol.Int. 34, 669-678
25. Sheth, B., Fesenko, I., Collins, J.E., Moran, B., Wild, A.E., Anderson, J.M., Fleming, T.P. (1997) Tight junction assembly during mouse blastocyst formation is regulated by late expression of ZO-1 a+ isoform. Development 124, 2027-2037
26. Andersen, C. L., Jensen, J. L., Orntoft, T. F. (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 64, 5245-5250
27. de Jong, P. M., van Sterkenburg, M. A., Kempenaar, J. A., Dijkman, J. H., Ponec, M. (1993) Serial culturing of human bronchial epithelial cells derived from biopsies. In Vitro Cell Dev.Biol.Anim 29A, 379-387
28. Ahmed, Z. M., Riazuddin, S., Ahmad, J., Bernstein, S. L., Guo, Y., Sabar, M. F., Sieving, P., Riazuddin, S., Griffith, A. J., Friedman, T. B., Belyantseva, I. A., Wilcox, E. R. (2003) PCDH15 is expressed in the neurosensory epithelium of the eye and ear and mutant alleles are responsible for both USH1F and DFNB23. Hum.Mol.Genet. 12, 3215-3223
29. Bouillot, S., Tillet, E., Carmona, G., Prandini, M. H., Gauchez, A. S., Hoffmann, P., Alfaidy, N., Cand, F., Huber, P. (2011) Protocadherin-12 Cleavage Is a Regulated Process Mediated by ADAM10 Protein: EVIDENCE OF SHEDDING UP-REGULATION IN PRE-ECLAMPSIA. J.Biol.Chem. 286, 15195-15204
30. Reiss, K., Maretzky, T., Haas, I. G., Schulte, M., Ludwig, A., Frank, M., Saftig, P. (2006) Regulated ADAM10-dependent ectodomain shedding of gamma-protocadherin C3 modulates cell-cell adhesion. J.Biol.Chem. 281, 21735-21744
31. Hulpiau, P., van Roy, F. (2011) New insights into the evolution of metazoan cadherins. Mol.Biol.Evol. 28, 647-657
32. Sheng, M., Sala, C. (2001) PDZ domains and the organization of supramolecular complexes. Annu.Rev.Neurosci. 24, 1-29
33. Blanco-Arias, P., Sargent, C. A., Affara, N. A. (2004) Protocadherin X (PCDHX) and Y (PCDHY) genes; multiple mRNA isoforms encoding variant signal peptides and cytoplasmic domains. Mamm.Genome 15, 41-52
34. Romagnoli, M., Vachier, I., Vignola, A. M., Godard, P., Bousquet, J., Chanez, P. (1999) Safety and cellular assessment of bronchial brushing in airway diseases. Respir.Med. 93, 461-466
35. Vignola, A. M., Chanez, P., Campbell, A. M., Bousquet, J., Michel, F. B., Godard, P. (1993) Functional and phenotypic characteristics of bronchial epithelial cells obtained by brushing from asthmatic and normal subjects. Allergy 48, 32-38

36. Gray, T. E., Guzman, K., Davis, C. W., Abdullah, L. H., Nettesheim, P. (1996) Mucociliary differentiation of serially passaged normal human tracheobronchial epithelial cells. Am.J.Respir.Cell Mol.Biol. 14, 104-112
37. Wu, R., Zhao, Y. H., Chang, M. M. (1997) Growth and differentiation of conducting airway epithelial cells in culture. Eur.Respir.J. 10, 2398-2403
38. Krahn, M. P., Rizk, S., Alfalah, M., Behrendt, M., Naim, H. Y. (2010) Protocadherin of the liver, kidney, and colon associates with detergent-resistant membranes during cellular differentiation. J.Biol.Chem. 285, 13193-13200
39. Ross, A. J., Dailey, L. A., Brighton, L. E., Devlin, R. B. (2007) Transcriptional profiling of mucociliary differentiation in human airway epithelial cells. Am.J.Respir.Cell Mol.Biol. 37, 169-185
40. Tanos, B., Rodriguez-Boulan, E. (2008) The epithelial polarity program: machineries involved and their hijacking by cancer. Oncogene 27, 6939-6957
41. de Boer, W. I., Sharma, H. S., Baelemans, S. M., Hoogsteden, H. C., Lambrecht, B. N., Braunstahl, G. J. (2008) Altered expression of epithelial junctional proteins in atopic asthma: possible role in inflammation. Can.J.Physiol Pharmacol. 86, 105-112
42. Kicic, A., Hallstrand, T. S., Sutanto, E. N., Stevens, P. T., Kobor, M. S., Taplin, C., Pare, P. D., Beyer, R. P., Stick, S. M., Knight, D. A. (2010) Decreased Fibronectin Production Significantly Contributes to Dysregulated Repair of Asthmatic Epithelium. Am.J.Respir.Crit Care Med.
43. Parker, J., Sarlang, S., Thavagnanam, S., Williamson, G., O'donoghue, D., Villenave, R., Power, U., Shields, M., Heaney, L., Skibinski, G. (2010) A 3-D well-differentiated model of pediatric bronchial epithelium demonstrates unstimulated morphological differences between asthmatic and nonasthmatic cells. Pediatr.Res. 67, 17-22
44. Stevens, P. T., Kicic, A., Sutanto, E. N., Knight, D. A., Stick, S. M. (2008) Dysregulated repair in asthmatic paediatric airway epithelial cells: the role of plasminogen activator inhibitor-1. Clin.Exp.Allergy 38, 1901-1910
45. Davies, D. E. (2009) The role of the epithelium in airway remodeling in asthma. Proc.Am.Thorac.Soc. 6, 678-682

SUPPLEMENTARY DATA

Supplementary methods: *Validation of PCDH1 isoform specific antibodies:* PCDH1 isoform specific antibodies were tested both on cell-lysates of L-cells overexpressing PCDH1 protein products and by blocking experiments. *Overexpression:* Mouse L-cell fibroblasts (kindly provided by Dr C. van Kooten, Leiden University Hospital, the Netherlands) were transfected with PCDH1 isoform 1 and isoform 2 contructs (TC109553 and TC109554, Origene, Rockville, USA) using Fugene 6 transfection reagent (Roche Diagnostics, Almere, the Netherlands). PCDH1 protein was detected in cell-lysates by western blotting using isoform specific antibodies (Figure E1A). *Blocking experiments:* Specificity of isoform specific antibodies was tested by blocking experiments with pre-incubation of ten-fold molar excess of immunizing peptide. Intensity of PCDH1 band at ~170 kDa was diminished in L-cells overexpressing full length PCDH1 isoform 1 or 2 or 16HBE after peptide pre-incubation, thereby proving the specificity of the antibodies (Figure E1B).

Table E1: Efficiencies of qRT-PCR assays as determined by cDNA dilution series

Brushed cells:			Cultured cells:		
Assay	Slope	Efficiency (%)	Assay	Slope	Efficiency (%)
Exon 1-2	-4,873	60,4	Exon 1-2	-4,0403	76,8
Exon 3-4	-5,1582	56,3	Exon 3-4	-4,5696	65,5
β-actin	-3,5666	90,7	Bact	-3,949	79,2
RPLPO	-3,7015	86,3	Rplpo	-3,849	81,9
β2M	-3,7005	86,3	b2m	-3,949	79,2
GAPDH	-3,6027	89,5	Gapdh	-3,6963	86,4

Table E2: Genbank accession numbers

Exons	Genbank accession nrs	Prevalence (%)*
Exon 1E - Exon 1	HO663511	2
Exon 1F - Exon 1	HO663512	84
Exon 1B - Exon 1	HO663513	4
Exon 1C - Exon 1	HO663514	2
Exon 1D - Exon 1	HO663515	8
Exon 3 - Exon 4	NM_032420	21
Exon 3 - Exon 4 with gap - Exon 5	HO663509	12
Exon 3 - Exon 4 - Exon 5	HO663510	61
Exon 3 - Exon 5	HO663509 - exon 4	6

** = Relative abundance of transcripts identified by 5'RACE and 3'RACE reactions*

Figure E1: Specificity of antibodies as determined by overexpression and blocking experiments.

Newly generated isoform specific polyclonal antibodies detect bands at ~150 kDa for isoform 1, and ~170 and ~50 kDa for isoform 2, in 16HBE and L-cells overexpressing isoform 1 or 2, while these bands were not detected using the control vector (eGFP) (A). Bands at ~170 kDa were completely blocked for isoformt 1 in 16HBE (16H), L-cells overexpressing isoform 1 (+) or asthma patient samples (P1-P4), when the antibody was pre-incubated with peptide. For isoform 2 both the ~170kDa and ~50 kDa were diminished in L-cells over-expressing PCDH1 isoform 2 and 16HBE, when the antibody was pre-incubated with immunizing peptide (B). NB=not blocked, FL=Full length.

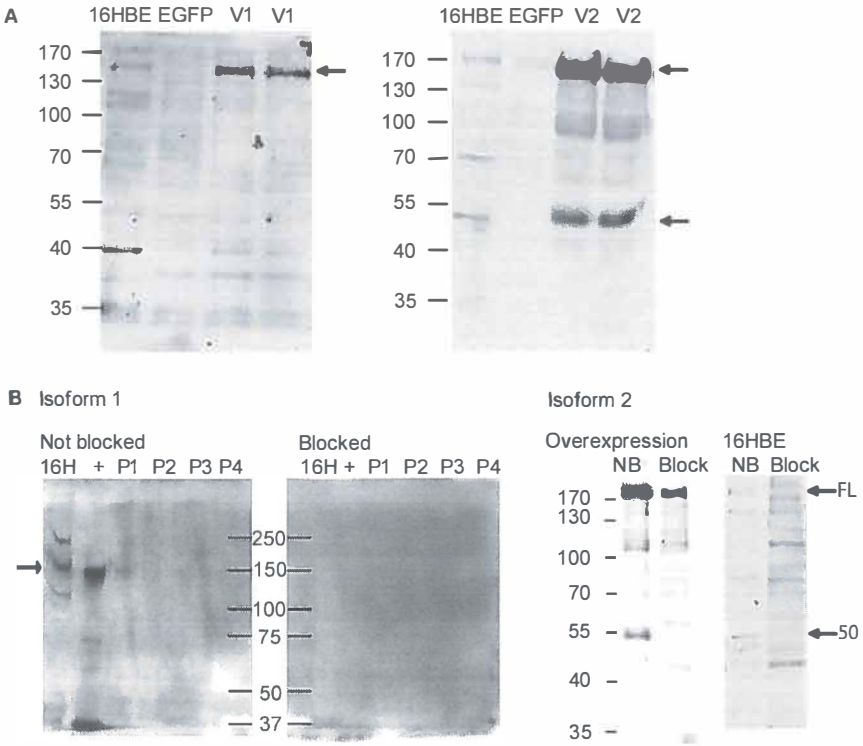
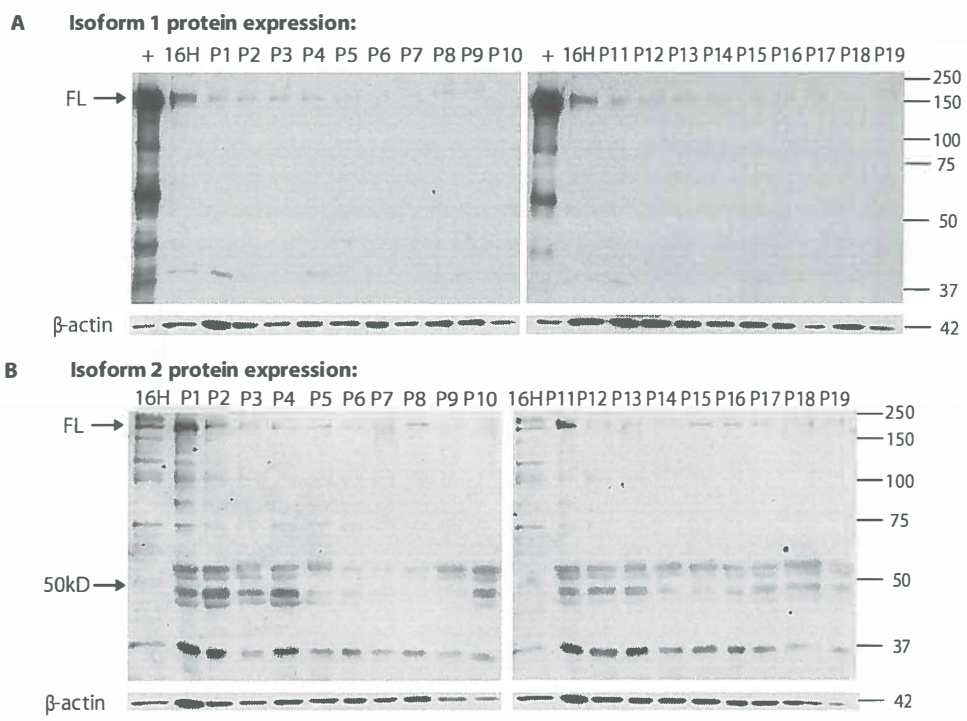


Figure E2: Isoform 1 and isoform 2 protein expression in cultured PBECs.

No or weak expression of PCDH1 isoform 1 is observed for asthma patient submerged cultured PBECs (P1 – P19), while the positive controls (+; L-cell overexpressing cells; 16H = 16HBE) give a clear signal (A). Low expression of PCDH1 isoform 2 was detected in cultured PBECs, as compared to 16HBE (B), while the 50kDa band was not detected in cultured PBECs. Arrows indicate the size of specific bands (~170kDa, isoform 1 and 2, ~50KDa isoform 2) previously detected by blocking experiments. FL = full length isoforms.



Chapter 5

Mouse Protocadherin-1 expression is regulated by house-dust mite and cigarette smoke exposure *in vivo*

Henk Koning
Antoon J.M. van Oosterhout
Uilke Brouwer
Lisette E. den Boef
Renée Gras
Sijranke Post
Marco van der Toorn
Machteld N. Hylkema
Ian Sayers
Gerard H. Koppelman
Martijn C. Nawijn

Submitted

ABSTRACT

Protocadherin-1 (*PCDH1*) is a novel susceptibility gene for airway hyperresponsiveness, and is expressed in bronchial epithelial cells. Here, we asked how mouse *Pcdh1* expression is regulated in lung structural cells *in vivo* under physiologic conditions, in a house-dust mite driven mouse model of asthma, and in short-term cigarette smoke exposure models, both characterized by airway hyperresponsiveness. *Pcdh1* gene-structure was investigated by Rapid Amplification of cDNA Ends. Pcdh1 mRNA and protein expression was investigated by qRT-PCR, western blotting and immunohistochemistry using isoform-specific antibodies. We observed 87% conservation of the *Pcdh1* nucleotide sequence, and 96% conservation of the Pcdh1 protein sequence between men and mice. We identified a novel *Pcdh1* isoform encoding only the intracellular signalling motifs. Airway epithelial cells expressed all Pcdh1 isoforms, while airway smooth muscle only expressed this novel isoform. Epithelial Pcdh1 protein expression was reduced in a chronic house dust mite induced asthma model, while its mRNA expression was induced in the resolution phase after house-dust mite challenges. Short-term cigarette smoke exposure also reduced *Pcdh1* mRNA expression in lung tissue (3 to 4-fold), while neutrophilia and airway hyperresponsiveness was induced. We conclude that *Pcdh1* is highly homologous to human *PCDH1*, encodes two transmembrane proteins and one intracellular protein, with distinct expression patterns in airway epithelial and airway smooth muscle cells, and is regulated by house-dust mite and cigarette smoke exposures *in vivo*.

INTRODUCTION

Asthma is a complex disease, caused by gene-gene and gene-environment interactions (1). Many asthma susceptibility genes have been identified, several of which are expressed in the airway epithelium (2). The airway epithelium in asthma has a disrupted barrier function (3) and an impaired repair response upon injury (4,5) that might contribute to airway remodelling. In addition, the airway epithelium of asthmatics shows an enhanced immune response towards harmful agents (allergens or viruses) by secreting increased amounts of pro-inflammatory mediators such as IL-33, CCL20, GM-CSF or TSLP (6-9). Polymorphisms in asthma susceptibility genes expressed by airway epithelial cells, affecting the level or regulation of their expression (10), might therefore contribute to both altered barrier function of airway epithelial cells and to enhanced induction of an immune response upon airway epithelial injury (11).

Previously, we identified protocadherin-1 (*PCDH1*) as a novel susceptibility gene for airway hyperresponsiveness (AHR) in asthma families (12). Interestingly, a strong linkage signal of *PCDH1* with asthma and AHR was observed in families exposed to environmental tobacco smoke (ETS). *PCDH1* encodes for two main isoforms: a 3 exon and a 5 exon isoform that are expressed in the airway epithelium (12). In addition a putative third isoform was identified that lacks exon 1 and part of exon 2 (13). Both main isoforms encode for a protein containing an extracellular domain with seven cadherin repeats, a transmembrane domain, and an intracellular domain containing several Serine and Tyrosine residues, that have been found to be subject to phosphorylation (14,15). The third isoform only contains two extracellular cadherin repeats and, as for the long 5 exon isoform, an intracellular domain containing three intracellular conserved motifs (CM1-CM3), of which CM3 is the binding motif for protein phosphatase 1 alpha (PP1α) (16,17). We previously reported complex splicing patterns of *PCDH1* regarding the expression of intracellular conserved motifs, and observed a marked upregulation of PCDH1 during mucociliary differentiation of primary bronchial epithelial cells (13).

In mouse, *Pcdh1* mRNA expression was identified in several adult tissues (brain, kidney, heart, lung and uterus), but highest expression was observed in lung (18). During mouse embryogenesis, *Pcdh1* mRNA expression in lung was restricted to mesenchyme and blood vessels, and was not detected in the bronchial epithelium. Similar to the human

situation, two main transcripts were identified in the mouse, as well as a variant displaying variation in expression of conserved motifs (16).

As *PCDH1* is a susceptibility gene for AHR and encodes an adhesion molecule that is expressed in the airway epithelium, we hypothesize that PCDH1 plays a role in the barrier function and repair of the epithelium. Currently, detailed knowledge about Pcdh1 expression in lung structural cells and its regulation by environmental exposures *in vivo* is unknown. Therefore, we aimed to investigate the expression and regulation of Pcdh1 under basal, damaging and repair conditions *in vivo* in asthma and smoke exposure mouse models, known to be associated with AHR.

MATERIAL AND METHODS

Animal Models

BALB/c mice (6 to 8 weeks) were purchased from Charles River Laboratories (L'Arbresle-Cedex, France), housed in individually ventilated cages, kept under specific pathogen-free conditions and maintained on a 12h light-dark cycle, with food and water ad libitum. Experiments were approved by the Institutional Animal Care and Use Committee of the University of Groningen (The Netherlands), and carried out following (inter-)national welfare regulations.

House Dust Mite (HDM) asthma models

Mild HDM-model: Female BALB/c mice (n = 4 per group) were exposed intranasally to 25µg HDM extract (Greer Laboratories, Lenoir, NC, USA) or PBS in 10 µl volume once a week for three weeks, followed by a two week rest period, and two consecutive intranasal exposures (Figure 3A). Lung lobes were snap frozen for RNA or protein isolation, filled with Tissue-Tek™ diluted in PBS (1:1) (Sakura Finetek Holland BV, Zoeterwoude, the Netherlands) and slowly frozen to -80°C, or embedded in paraffin.

Chronic HDM-model: Male BALB/c mice were exposed intranasally with 25µg of HDM extract (Greer) (n = 5) or PBS (n = 4) in 10 µl volume twice a week for five weeks as described previously (Figure 4A) (19). Lung lobes were embedded in paraffin for Pcdh1 immunohistochemistry, 24h after the last HDM exposure.

Cigarette smoke (CS) exposure models

BALB/c mice were exposed to gaseous-phase CS from Kentucky 3R4F research reference cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, USA). Each cigarette was smoked without filter in 5 minutes at a rate of 5 L/hr in a ratio with 60L/hr air using whole body exposure. Gaseous-phase CS was directly distributed inside 6-liter perspex boxes. In this study we employed two CS-exposure models:

Sub chronic CS model: Female mice (n = 8 per group) were exposed to CS of 1-5 cigarettes for 5 days or filtered air each morning and afternoon, using a peristaltic pump as described previously (20). Mice were sacrificed 2h after the last CS exposure (Figure 5A).

Acute CS model: Male mice were exposed to CS of 10 cigarettes (n = 8) in 1.5h or filtered air (n = 5), and were sacrificed 6h after the last CS exposure (Figure 6A).

From all mice, Bronchial Alveolar Lavage (BAL)-fluid was isolated and the smallest lung lobe was stored at -80°C for RNA or protein isolation. BAL was obtained, by lavaging through a tracheal cannula with five 1 ml aliquots of saline of 37°C. Differential BAL cell counts (3 times 100 cells) were obtained from cytospin preparations stained with Diff-Quick (Merz & Dade A.G., Dudingen, Switzerland).

Airway responsiveness (AHR) was assessed in a separate group of sub chronic exposed mice at day 5, by omitting the final smoke exposure (n = 8 smoke exposed, n = 8 air exposed). AHR was determined by measuring airway resistance in response to i.v. administration of increasing doses of methacholine (acetyl-b-methylcholine chloride, Sigma-Aldrich, St. Louis, MO), using a computer-controlled small-animal ventilator (Flexivent; SCIREQ, Montreal, Quebec, Canada) as described previously (19).

RNA purification, Rapid amplification of cDNA ends (RACE) and quantitative (q)RT-PCR.

RNA was isolated from 50-100 mg of mouse lung tissue homogenized in 1 ml of TriReagent (MRC, Cincinnati, OH). gDNA traces were removed enzymatically, followed by purification using RNeasy Mini Kit (QIAGEN). RNA concentration and integrity was determined by Nanodrop measurements (ND-1000 spectrophotometer, Isogen Lifesciences, de Meern, Netherlands).

RACE was performed on RNA purified from lung tissue according to the manufacturer's instructions (GeneRacer-RLM-RACE Kit, Invitrogen, Carlsbad, USA), and as described previously (13) using the primers described in Table 1. RACE was performed from Pcdh1 exon 1 for 5' transcripts and from exon 3 for 3'transcripts. Furthermore, additional

5'RACE experiments from exon 3 were performed in order to identify a homolog of the putative human isoform 3 (13). PCR products were TOPO-cloned and *Pcdh1* transcripts were sequenced (StarSEQ®, Mainz, Germany) as described previously (13). Pcdh1 mRNA and protein sequences were compared to human *PCDH1* using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Expression levels of *Pcdh1* transcripts were determined by qRT-PCR as described previously (13), using ABI primer-probe sets. In brief, two microgram of RNA was reverse transcribed in 20µl reaction volume using Omniscript™ reverse transcriptase (QIAGEN Benelux BV, Venlo, the Netherlands). *Pcdh1* exon 1-2 (Mm01264041_m1, Applied Biosystems Europe BV, Nieuwekerk A/D IJssel, the Netherlands), *Pcdh1* exon 3-4 and *Pcdh1* exon m-1B (Custom assays, Applied Biosystems, Table 1 for primer sequences) expression was determined relative to the most stable combination of four housekeeping genes (*HPRT1* (Mm01545399_m1), *Pgk1* (Mm01225301_m1), *B2m* (Mm00437762_m1), *Ipo8* (Mm01255158_m1), Applied Biosystems). Data was analyzed using SDS2.3 software by applying the ΔΔCt-method (Applied Biosystems User Bulletin 2). As four house-keeping genes were used, for normalization the best combination of house-keeping genes was determined by using the Normfinder applet (21). We calculated relative expression levels as follows: First the cycle threshold value (Ct-value) of the average of the combination of most stable house-keeping genes was subtracted from the Ct-values of our Pcdh1-assays (formula 1). Next, we converted the ΔCt-value to expression level (EL; formula 2), and normalized the expression levels relative to the average of the control group (formula 3). In formulas: 1). $\Delta Ct_{PCDH1} = Ct_{PCDH1} - Ct_{HKG\text{-}average}$; 2). $EL_{PCDH1} = 2^{-\Delta Ct PCDH1}$; 3). Relative EL = EL_{PCDH1} / $EL_{PCDH1\text{-}control}$.

Table 1: Pcdh1 RACE and qPCR primer sequences

Pcdh1 RACE primer	Sequence (5'-3')	Corresponding GeneRacer primer	Sequence (5'-3')
5'RACE 301Rev	GAGGTCTCTGTGGTGAAAATGTCT	GeneRacer 5' primer	CGACTGGAGCACGAGGACACTGA
5'RACE 162Rev	GTTCCTCTGGCACCTTGTATACTACC	GeneRacer 5' nested primer	GGACACTGACATGGACTGAAGGAGTA
3'RACE FW1	GCCCAGGAGCTGCAGGATCCAT	GeneRacer 3' primer	GTCGTCAACGATACGCTACGTAACG
3'RACE FW2	GAAACACCCTCTAGCAAGTCATCCT	GeneRacer 3' nested primer	CGCTACGTAACGGCATGACAGTG
5'RACE Ex3 Rev3	GCTGTGATGGATCCTGCAGCTCCT	GeneRacer 5' primer	CGACTGGAGCACGAGGACACTGA
5'RACE Ex3 Rev2	ACTGGTGGCCGAGAAGGTGACA	GeneRacer 5' nested primer	GGACACTGACATGGACTGAAGGAGTA
qPCR assay	Forward (5'- 3')	Probe (FAM-NFQ labelled)	Reverse (5'-3')
M-1B-Ex1	CTCAGCATGCGCAGAAGAAAA	ACTGTTCTCCTGATTCTGG	CCTCAGAGGCCCCATCCT
Ex2-Ex3	GAGCAGTACTCCGACTACAG	CCAGCAAGCAGTTACCTCAC	TAGAGGGTGTTTCCGATTCC

Ex = exon; Rev = reverse; FW = Forward

Cloning and overexpression of Pcdh1 isoform 3 in BEAS2B bronchial epithelial cells

Cloning: The human DNA sequence corresponding to the isoform 3 sequence obtained by RACE PCR experiments, was amplified from the isoform 2 plasmid (pCMV-XL6-iso2, TC109554, Origene, Rockville, USA) with forward primer CCGCGAATTCATTCTCTTTGGTGTGGTGG and reverse primer CACTGGAGTGGCAACTTC by using Phusion™ High Fidelity PCR Kit (F-553S, Finnzymes, Fisher Scientific, Landsmeer, The Netherlands), followed by subcloning of the PCR product into the pCMV-XL6 expression vector (Origene, USA). The resulting pCMV-XL6-Pcdh1-Iso3 plasmids were sequenced (StarSeq Sequencing, Mainz, Germany).

Overexpression: BEAS2B cells were grown as described previously (22), seeded into 24-well plates, and transfected at a confluency of 60-80% with 0.5µg of eGFP (expression control, pEGFP-C1, Westburg BV, Leusden, The Netherlands), full-length Isoform 2 (pCMV-XL6-Pcdh1-iso2), or Isoform 3 plasmid (pCMV-XL6-Pcdh1-iso3), using Fugene HD transfection reagent according to the manufacturer's procedures (Roche Diagnostics, Almere, the Netherlands). Cells were harvested 48h after transfection using leammli buffer (2x leammli: 4% SDS; 20% glycerol; 10% β-mercaptoethanol; 0.004% bromphenol blue; 0.125 M Tris HCl; pH6.8), boiled for 5 min, and analysed for PCDH1 protein expression by western blotting using the antibody specific for the intracellular domain encoded by exon 4 (EP76, Figure 1A) (13).

Detection of Pcdh1 protein expression levels by western blotting

Protein isolation: Frozen lungs were homogenized in 5µl/mg lysis buffer (1% Triton-X 100, 150mM NaCl, 5mM $MgCl_2$, 10mM HEPES, 1:500 Protease Inhibitor Cocktail (P8340, Sigma-Aldrich, Zwijndrecht, the Netherlands)) using a tissue homogenator (T10 basic, ULTRA-TURRAX, IKA®, Staufen, Germany). Subsequently lung homogenates were boiled for 10 minutes with 2x laemmlli buffer and stored at -80°C.

Western blotting: Lung tissue homogenates were separated on 7.5% acrylamide SDS-page gels (Bio-Rad Laboratories BV, Veenendaal, the Netherlands). Detection of Pcdh1 protein was performed using antibodies that were designed against specific peptide sequences in the intracellular tail of both human *PCDH1* variants as described previously (13) (Figure 2A). These antibodies showed cross-reactivity with mouse *Pcdh1* due to high homology of the immunizing peptide sequences: (QPFQLSTPQPLPHPYH, EP78, 86% conserved; SPSPPEDRNTKTAPV, EP76, 100% conserved) (See Figure 1A for binding sites of antibodies). Antibodies were affinity column purified against immunizing peptides

(Eurogentec, Liege, Belgium), and were validated by blocking experiments using a pre-incubation period of 30 min at 37°C with 10x molar excess of immunizing peptides or H_2O.

Pcdh1 bands on western blots were visualized using Goat-anti-Rabbit-Peroxidase (GARPO, Daco, Heverlee, Belgium) as secondary antibody, and as tertiary step Rabbit-anti-Goat-Peroxidase (RAGPO, Dako, Heverelee, Belgium) was used. Next, blots were incubated with Luminol-reagent, and subsequently analysed using Fuji Medical X-Ray films (Fuji Photo Film Gmbh, Germany), followed by densitometric analysis using Quantity One Software v4.6.2 (Bio-Rad), relative to the β-actin loading control (sc-47778, Santa-Cruz Biotechnology, Heidelberg, Germany).

Immunohistochemistry

Periodic Acid Schiff (PAS) staining was performed on 3µm thick paraffin sections. Eosinophils were detected by cyanide resistant eosinophil peroxidase reaction on 5 µm thick frozen lung sections (23). Pcdh1 immunohistochemistry on paraffin embedded lung tissue was performed as follows: 3 µm thick paraffin sections were deparaffinized and antigen retrieval was performed by 30 min incubation with Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH=9.0) at 100°C. Pcdh1 specific antibodies, GARPO, RAGPO and AEC (3 amino-9-ethylcarbazole (Merck, Darmstadt, Germany)) were used to visualize Pcdh1 protein. Tissue was counterstained with haematoxylin (Merck, Darmstadt, Germany). Images were obtained with Cell^B Olympus imaging software using an Olympus BX50 microscope with DP70 camera. Pcdh1 epithelial staining intensity was determined using ImageJ software (http://rsb.info.nih.gov/ij/). In brief, background intensity values were determined in open spaces in the airway. Next, airway epithelial staining was determined by specifically selecting the epithelial layer. Relative intensity values were obtained by subtracting the background intensity values from the epithelial intensity values.

Statistical analysis

Differences in mRNA or protein expression levels were statistically tested by Mann-Whitney tests (GraphPad v4.0, La Jolla, USA). Differences in airway hyperresponsiveness between smoke-exposed and air-exposed mice were determined by testing for differences in the area under the curve (AUC) using GraphPad software.

RESULTS

Pcdh1 expression in the mouse lung

To allow an accurate investigation of *Pcdh1* expression regulation in the mouse lung, we first determined the *Pcdh1* gene-structure by RACE experiments. Compared to the published mouse *Pcdh1* gene-structure (16), we detected two novel exons on the 5' end of *Pcdh1* (exon m-1b and m-1c, Figure 1A), two novel exons on the 3'end (exon 3b and exon 5), as well as major variation in exon 4, containing the conserved CM2-domain and PDZ-BS motifs (Figure 1A). Exon 3b is a short 39 basepair exon encoding a premature stop codon, which is completely conserved between men and mice (See Table 2 for corresponding Genbank accession numbers).

Table 2: Genbank accession numbers of novel Pcdh1 transcripts

RACE reaction	*Protocadherin-1* Sequence	GenBank Accession number
Mm 5'RACE exon 1	Exon m-1b - 1	JK707005
Mm 5'RACE exon 1	Exon m-1c - 1	JK707006
Mm 5'RACE exon 1	Exon m-1c - 1 (short exon)	JK707007
Mm 3'RACE exon 3	Exon 3-3b-4	JK707008
Mm 3'RACE exon 3	Exon 4 including gap	JK707009
Mm 3'RACE exon 3	Exon 4-5	JK707010
Mm 5'RACE exon 3	Exon 2-3 (Isoform 3)	JK707011
Hs 3'RACE exon 3	Exon 3-3b-4-5	JK707004

Mm = Mus musculus; Hs = Homo sapiens

Remarkably, mouse *Pcdh1* exons had high homology to human exons resulting in a 87% homology at the nucleotide level (Figure 1A) and a 96 % homology on protein level (Supplementary Figure E1). Previously we detected an isoform lacking several extracellular domains in human bronchial epithelial cells (13). We therefore performed 5'RACE experiments from exon 3 in mouse lung. Interestingly, we identified an additional transcript starting from within exon 2, containing a CTG Kozak-sequence (isoform 3, Genbank accession JK707011), and putatively encoding a protein product with an in-frame translation initiation at amino-acid no 870 of the full-length PCDH1 isoform 2 protein (Genbank accession NP_115796). Overexpression of the corresponding 5'-truncated open reading frame in BEAS2B cells induced 40-and 50kD Pcdh1 proteins. Both protein products were specifically detected by the EP76-antibody as intensity of these bands clearly diminished after blocking with immunizing peptide, and were not detected with the EP78 and EP209 antibodies (Figure 1B). These results show that in mouse lung a third, intracellular Pcdh1

isoform is expressed that does not contain the extracellular and transmembrane domains, but retains all intracellular domains also present in Pcdh1 isoform 2 (Figure 1B).

We next investigated basal expression of *Pcdh1* mRNA and protein levels in mouse lung tissue. We observed very low levels of *Pcdh1* using the exon m-1b/exon-1 specific qRT-PCR assay, while much higher expression levels of *Pcdh1* were detected using exon 1-2 and exon 3-4 specific assays (Figure 2A). On western blot of whole lung homogenate, we observed two specific bands of 140-and 150 kD using a specific antibody generated against the intracellular tail of the Pcdh1 isoform 1 (EP78, Figure 2B). In contrast, we observed a 50kD and 170kD bands using a specific antibody generated against the intracellular tail present in Pcdh1 isoforms 2 and 3 (EP76, Figure 2B).

Immunohistochemical staining revealed expression of Pcdh1 in the airway epithelium, using three antibodies that detect either intracellular (EP76 and EP78) or extracellular (EP209) Pcdh1 domains (Figure 1A and 2C). In addition, smooth muscle cells surrounding airways and blood vessels stained positive for Pcdh1, but only with antibody EP76 (Figure 2C). These results suggest that airway smooth muscle cells (ASMs) express an N-terminally truncated Pcdh1 protein containing intracellular, but not extracellular domains, consistent with the novel isoform 3.

Figure 1: Pcdh1 gene-structure and isoforms

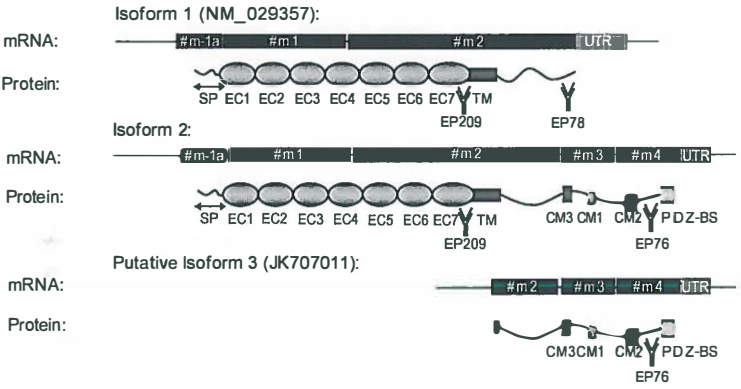
Two novel exons were detected at the 5'end of PCDH1, and one novel exon on the 3'end of Pcdh1. Mouse Pcdh1 exons share high homology with human PCDH1 exons. Corresponding isoforms (isoforms 1, 2 and putative isoform 3) and their protein structures are depicted. Only isoform 2 and putative isoform 3 contain evolutionary conserved motifs (CM1-3). Two antibodies were generated against sequences in the intracellular tail of both isoform 1 (EP78) and isoform 2 (EP76) of Pcdh1. An antibody directed against the extracellular domains of Pcdh1 (EP209) was generated previously (12) *(A). Western blot of lysates of BEAS2B cells overexpressing the open reading frame of Pcdh1 isoform 3, using antibody EP76 for detection as indicated (left hand panel) including a pre-incubation with the immunizing peptide ('Peptide blocked') as a control for specificity (right hand panel). Western blots using EP78 and EP209 antibodies for detection are also included as indicated (B). cds = coding sequence; UTR = Untranslated region; SP = Signal peptide (amino-acid (aa) 1-57); EC = Extracellular Cadherin domain; TM = Transmembrane domain; CM = Conserved Motif; PDZ-BS = PDZ-domain Binding Site; EP209 = Binding site for antibody directed against the extracellular domain of Pcdh1; EP78 = Binding site for antibody directed against a specific intracellular sequence of isoform 1, encoded by exon 2; EP76 = Binding site for antibody directed against the intracellular domain present in isoforms 2 and 3 and encoded by exon 4; FL = full length. Genbank accession numbers of novel transcripts are provided in Table 2.*

A Pcdh1 gene-structure

Homology

Mouse exons	m-1a	m-1b	m-1c	1	2cds	2+UTR	3	3b	4cds	4+UTR	5
Size (basepair)	182	153	211	863	2280	2835	220	39	398	1055	100
Human exons	1a	Na	1e/1f	1	2cds	2+UTR	3	3b	4cds	4+UTR	5
Size (basepair)	180	Na	195	863	2280	2776	220	39	395	1063	146
Homology (%)	87	Na	73	87	89	86	89	100	96	87	97

Protein structure



B Overexpression of isoform 3 in BEAS2B

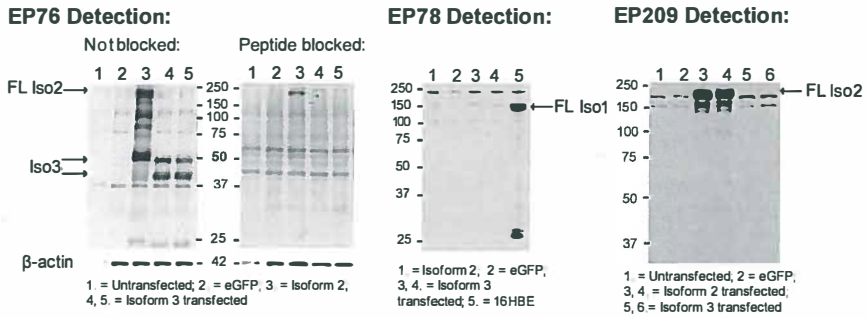
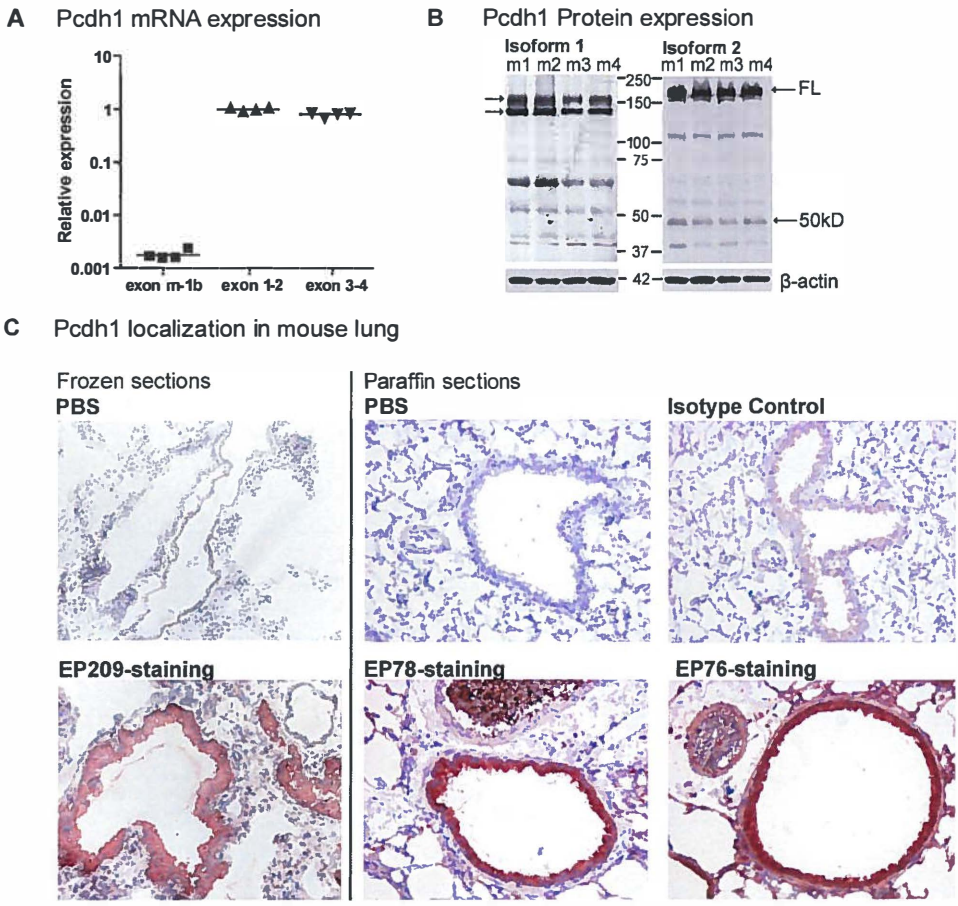


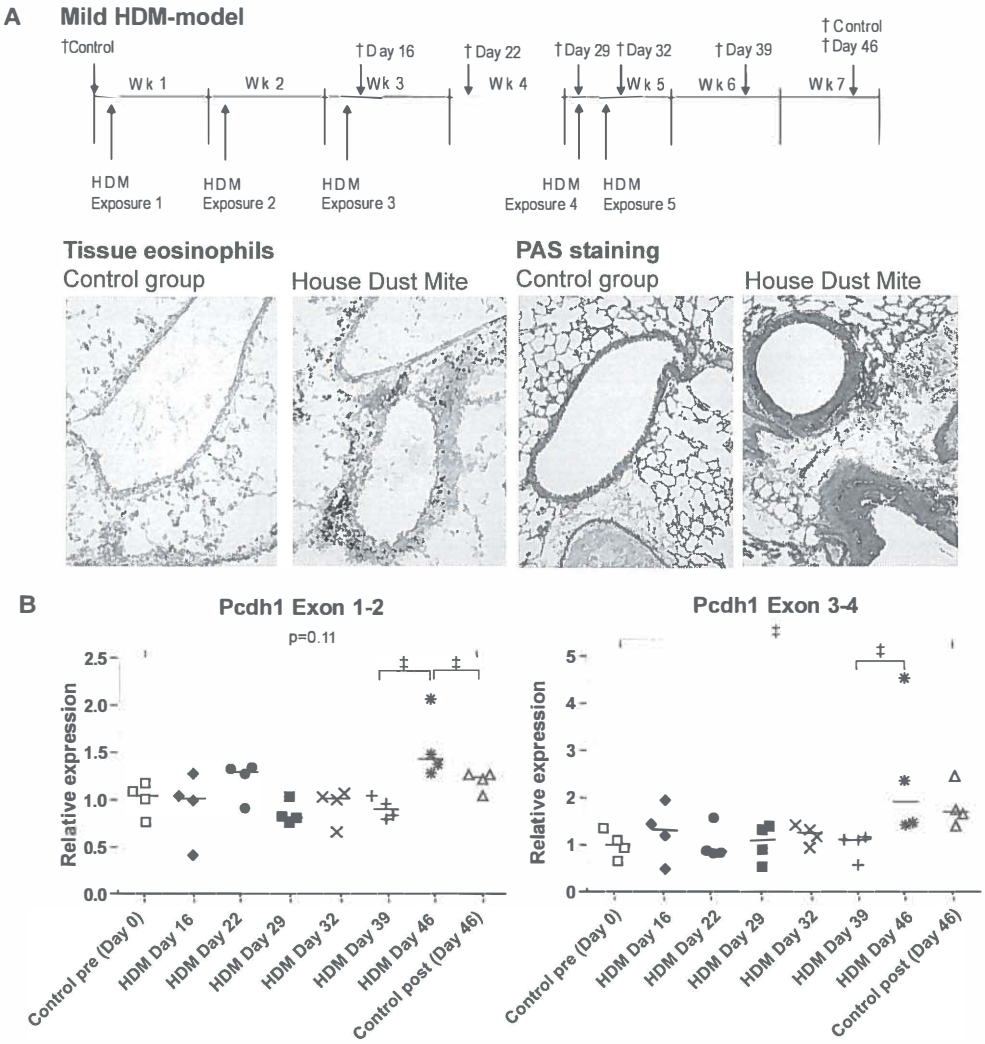
Figure 2: Basal Pcdh1 mRNA and protein expression, and localization.
mRNA expression levels of exon m-1b, exon 1-2 and exon 3-4 containing transcripts in naive mouse lung of four mice (A). Western blot of mouse lung homogenate using the antibody directed against the intracellular domain of Pcdh1 isoform 1 (EP78), and the antibody directed against the intracellular domain encoded by exon 4 of Pcdh1 (isoform 2, EP76) (see arrows), with β-actin as loading control (B). Immunohistochemical staining of Pcdh1 on frozen sections using the 209-antibody and on paraffin sections using antibodies EP78 and EP76. PBS and IgG (isotype) were used as controls (C). m1 = lung homogenate of mouse number 1; FL = full length.



Pcdh1 expression levels in HDM driven asthma models

Previously, we observed PCDH1 expression in the human airway epithelium (12), and here we confirmed epithelial expression in the mouse lung. We aimed to identify whether Pcdh1 expression is altered in house-dust mite (HDM) driven asthma models, as these models of asthma are induced by sensitisation through the airway epithelium, resulting in AHR, eosinophilic airway inflammation, goblet cell metaplasia and increased ASM-mass in the airways as well as the induction of HDM-specific IgE responses (24). We first employed a 3-week HDM-exposure model based on Hammad and co-workers (25), which induced eosinophilic inflammation and goblet cell metaplasia in the airways (Figure 3A). To test whether Pcdh1 was involved in subsequent repair, we allowed the mice to recover for 2 weeks followed by an additional 2 HDM exposures (exposure 4 and 5, Figure 3A) and another recovery period. In the control (PBS-exposed) groups terminated prior to (day 0) and at the end of the experiment (day 46), we observed a trend towards higher expression of exon 1-2 ($p=0.11$), and a significant higher expression of exon 3-4 ($p<0.05$) during the 7-week period of the experiment (Figure 3B). We observed no alterations in *Pcdh1* expression during the first 3 weeks of HDM exposure, during the two weeks of recovery thereafter, or during the second series of HDM exposures (Figure 3B). However, we observed a clear upregulation of *Pcdh1* mRNA expression between 7 and 14 days after the last two HDM exposures, for both exon 1-2 and exon 3-4 assays (HDM day 39 vs HDM day 46, $p<0.05$ for both assays) (Figure 3B). Our design did not enable direct comparison to a control group at this time-point. Since *Pcdh1* expression levels of the control groups were higher at the end than at the start of this HDM-model, it is hard to conclude whether the marked *Pcdh1* expression difference reflects a reduced expression of *Pcdh1* during the two consecutive HDM exposures, or a strong induction of expression during the resolution phase thereafter. Furthermore, no differences in protein levels were detected between the different groups when analysed by western blot or immunohistochemistry (data not shown). Therefore, we performed a second experiment in which we compared Pcdh1 expression between HDM- and PBS treated mice that had received a higher number of HDM exposures (Figure 4A). In this model we observed marked eosinophilic airway inflammation, airway remodelling and AHR as published previously (19). Immunohistochemical staining for Pcdh1 isoforms revealed strong staining in intact epithelial cell layers using isoform 1 and isoform 2/3-specific antibodies. In contrast, no (isoform 1-specific antibody) to low (isoform 2/3-specific antibody) staining was observed in remodelled airways characterized by goblet cell metaplasia (Figure 4B).

Figure 3: Expression of Pcdh1 after HDM-exposure in the mild HDM-model
In the mild HDM model mice are exposed to HDM (25µg in 10µl PBS) or PBS (control) once a week for three weeks, followed by a two week rest period and two consecutive exposures (exposures 4 and 5). After these two exposures two additional weeks of rest are included. This model is characterized by tissue eosinophilia and goblet cell hyperplasia (A). mRNA expression levels of Pcdh1 exon 1-2 and 3-4 were determined during HDM exposures (closed symbols), compared to the control group (open symbols) (B). Wk = week; HDM = house-dust mite; † = day at which mice are sacrificed; PAS = Periodic Acid Schiff-staining for mucous of goblet cells; exp. = exposure; ‡ = p<0.05.



To quantify the differences of airway epithelial Pcdh1 expression between PBS and HDM-exposed mice we next determined the mean intensity of Pcdh1 expression in intact epithelial cell layers, not affected by goblet cell metaplasia. Pcdh1 isoform 1 expression was the same in both groups while, in contrast, lower expression levels of Pcdh1 isoform 2/3-specific staining was observed (p=0.035) in HDM exposed mice when compared to PBS exposed controls (Figure 4C). These data indicate that in the active phase of HDM-induced airway inflammation, expression of Pcdh1 isoform 2 and/or isoform 3 was lower in airway epithelial cells.

Short term smoke exposure down regulates Pcdh1 expression levels

Given the initial linkage of *PCDH1* to AHR and asthma in cigarette smoke exposed families defined by a significant smoking history of the asthma proband of more than 5 packyears (12), we investigated whether *Pcdh1* expression was regulated in a sub chronic (5 days) smoke exposure model (Figure 5A). This model induced a neutrophillic influx into the airways (Figure 5A), a feature of sub chronic smoke-exposure models (20, 26), as well as a trend towards AHR to methacholine (p=0.056, Figure 5A).

In CS-exposed mice, we observed significantly lower expression of *Pcdh1* transcripts encoding exon 1-2 (3.9 fold, p=0.0003) and exon 3-4 (3.3-fold, p=0.0003) in total lung (Figure 5B). Next, we investigated whether this was reflected at the protein level in total mouse lung homogenate. No differences in Pcdh1 isoform 1 protein levels were found, but a clear trend towards lower levels of the 170 kd Pcdh1 isoform 2 protein band (p=0.052, Figure 5C and 5D) was observed by western blot after 5 days of smoke exposure.

Figure 4: Expression of Pcdh1 after HDM exposure in the 5-week HDM-model
In this HDM model mice are exposed to HDM (25μg in 10μl PBS) or PBS (control) twice a week for 5 weeks as described previously (19) *(A). Pcdh1 protein expression levels are depicted of HDM-exposed airways with and without goblet cell metaplasia, and in PBS-exposed airways, using EP78 and EP76 antibodies (B). Pcdh1 protein levels of EP78 and EP76-stainings were quantified in intact HDM exposed airways (closed symbols) compared to PBS exposed airways (open symbols), by comparing the relative intensity levels. IgG-isotype was used as control (C). Exp. = exposure; Wk = week; HDM = house-dust mite; † = day at which mice are sacrificed.*

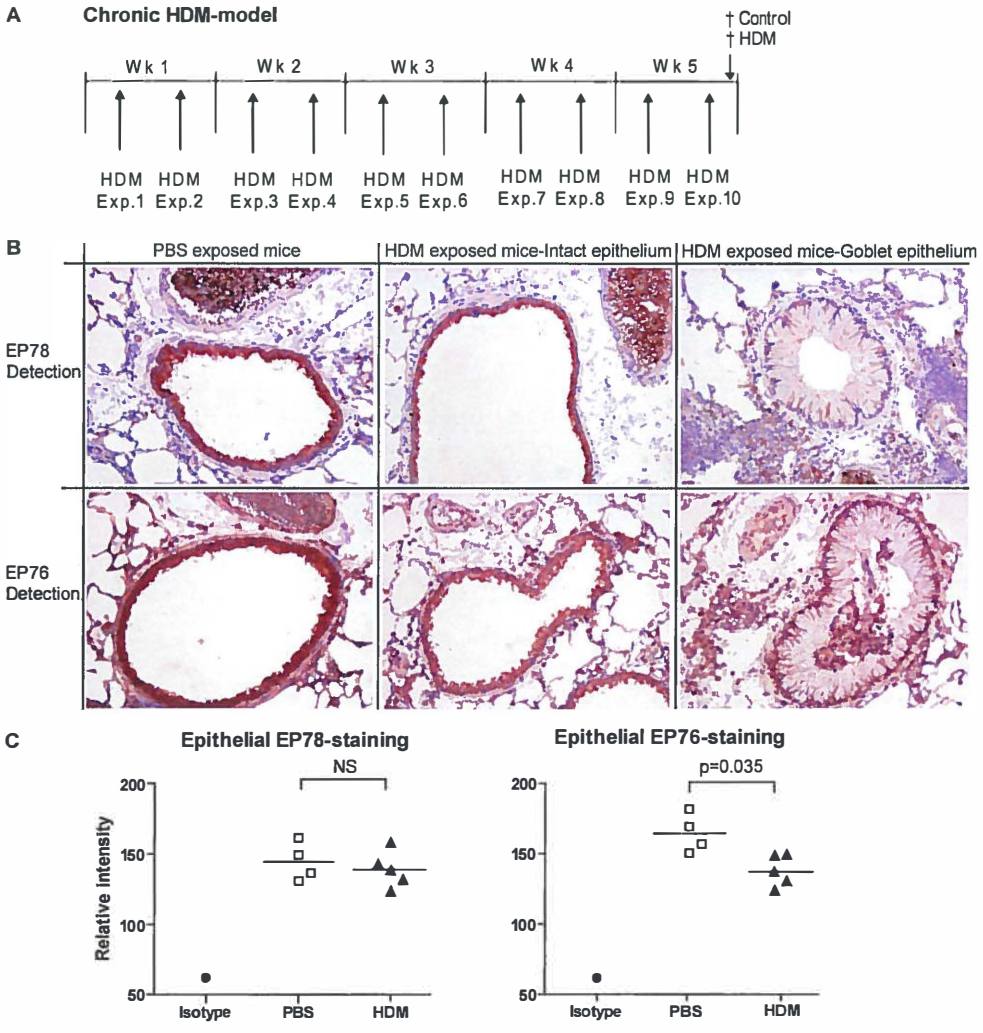
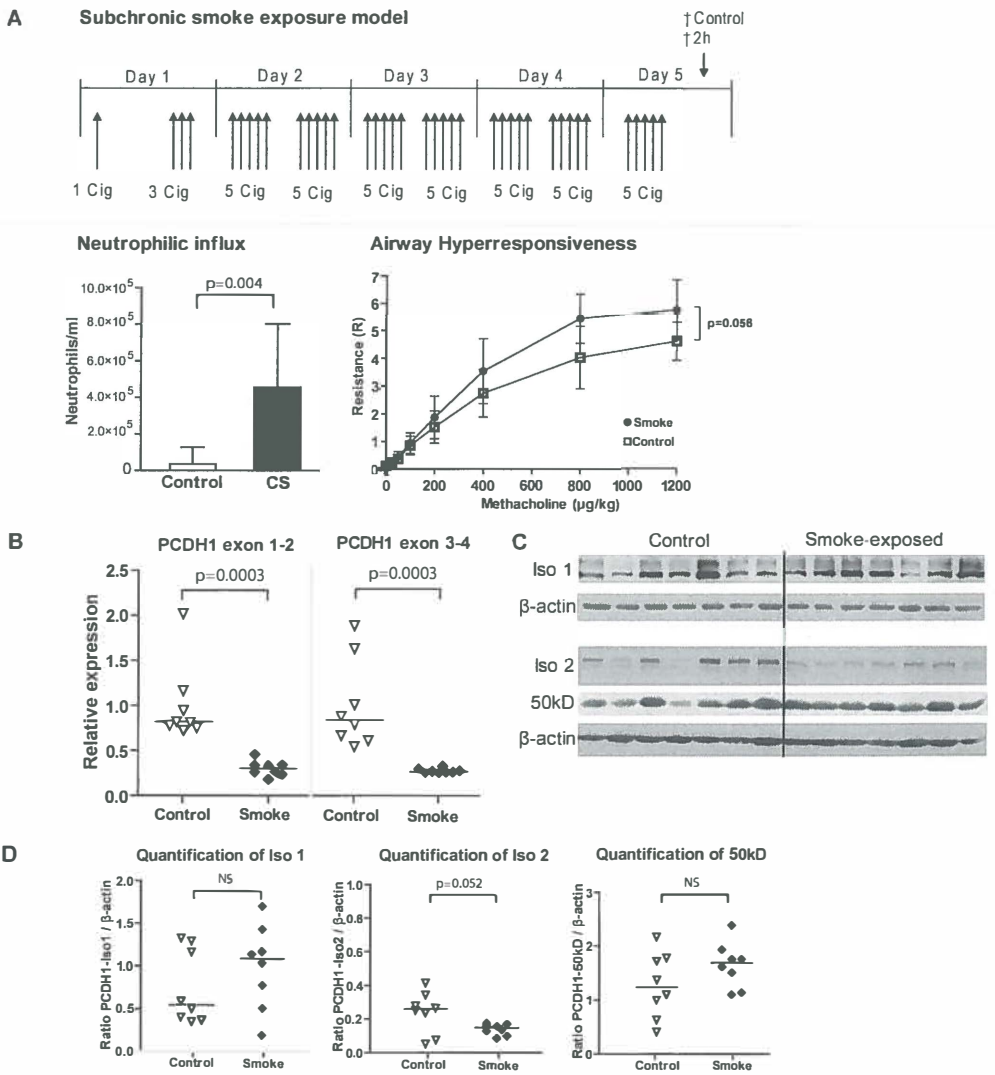


Figure 5: Pcdh1 expression levels decrease after sub chronic smoke exposure
In the sub chronic smoke exposure model, mice were exposed to 1 and 3 cigarettes or air at day 1, followed by 5 cigarettes at day 2-5 or air. 2h after the last cigarette both smoke and air exposed mice were sacrificed. Neutrophils in the bronchoalveolar lavage fluid, and airway hyperresponsiveness to methacholine were determined. Error bars show Standard Deviation (A). Pcdh1 exon 1-2 and 3-4 transcript levels were determined in lungs of air exposed (open symbols) and smoke exposed (closed symbols) mice (B). Pcdh1 protein levels were determined by western blot of lung homogenate of air (control) and smoke exposed mice. Pcdh1 isoform 1 (EP78-antibody), full-length isoform 2 (EP76-antibody) and 50kD (EP76-antibody) levels are shown, with β-actin as loading control (C). Densitometric quantification of Pcdh1 expression levels of full-length isoform 1 and 2, and the 50kD fragment, relative to β-actin (D). Cig = cigarette; † = time-point at which mice are sacrificed.

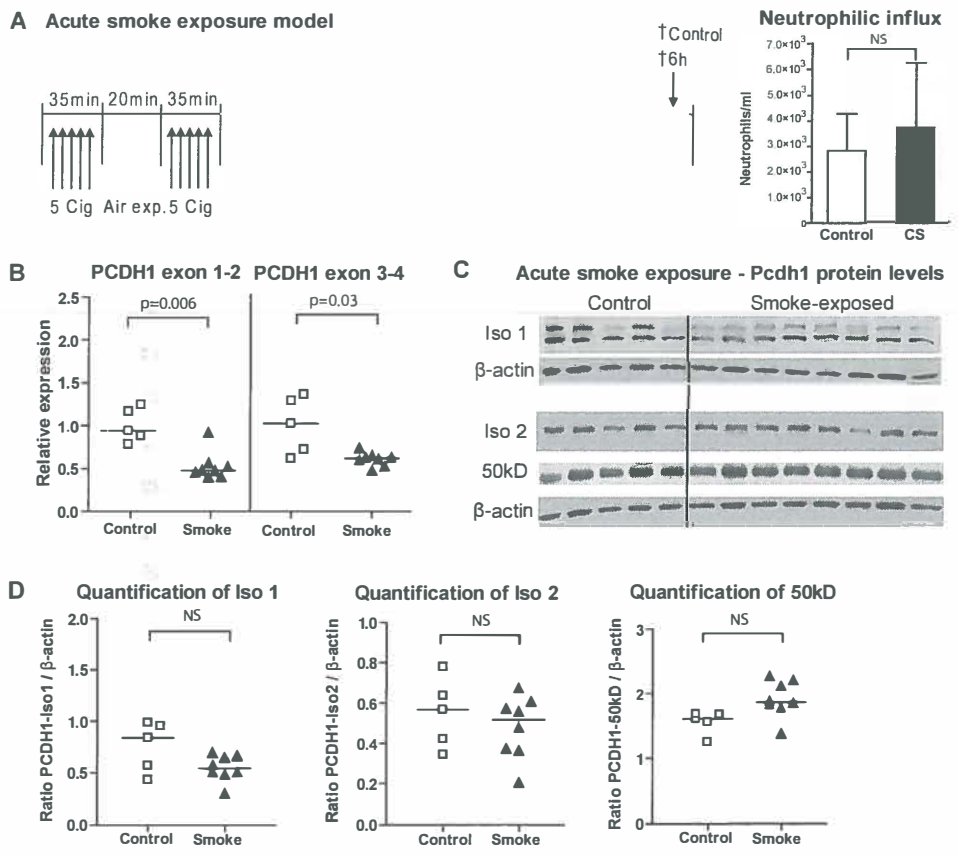


To test whether this down regulation of Pcdh1 expression levels was a direct effect of CS-exposure, we also analyzed Pcdh1 expression shortly after an acute CS-exposure, in which no significant neutrophilic influx was observed (Figure 6A). Remarkably, already 6 hours after CS-exposure, a 2-fold (p=0.006) decrease in exon 1-2 expression levels and a 1.6-fold (p=0.03) decrease in exon 3-4 expression levels was observed (Figure 6B), suggesting that *Pcdh1* expression indeed is directly regulated by CS-exposure. This difference was not paralleled by a reduced lung Pcdh1 protein expression at this time-point (Figure 6C and 6D).

DISCUSSION

In this study, we provide a full characterization of the expression pattern of a novel asthma and AHR susceptibility gene in the mouse lung, and its regulation in relevant *in vivo* models known to modulate AHR by direct effects on the airway epithelium. Here, we show that the mouse and human genes for *Protocadherin-1* are highly homologous, both at the nucleotide and at the amino-acid level, validating the mouse as a relevant model to study Pcdh1 function. As seen in human bronchial biopsies (12), Pcdh1 is mainly expressed in the airway epithelium. In addition to the two annotated isoforms of Pcdh1 that differ in their cytoplasmic signalling domains, we observed expression of a novel Pcdh1 isoform, characterized by the lack of extracellular and transmembrane domains, whilst containing the intracellular domains also present in isoform 2. With immunohistochemistry, we found a staining pattern consistent with the presence of this novel isoform, specifically in smooth muscle cells surrounding the airways and venules in the lung. In the active phase of both house-dust mite and sub chronic cigarette smoke exposure models at the time of AHR, Pcdh1 expression levels were at the lowest. Specifically, epithelial Pcdh1 protein expression was reduced after chronic exposure to house-dust mite in an asthma model, while in the resolution phase after house-dust mite exposure an induction of *Pcdh1* mRNA expression was observed. Interestingly, acute exposure of mice to CS induced a rapid and strong reduction of Pcdh1 expression.

Figure 6: Pcdh1 mRNA expression levels decrease after acute smoke exposure
In the acute smoke exposure model, mice were exposed to 10 cigarettes, with a 20 min rest period in between, or air. Low numbers of neutrophils were observed in the lung after 6h (A). Pcdh1 exon 1-2 and 3-4 mRNA expression levels were determined in the smoke exposed group (closed symbols) and compared to the air-exposed group (open symbols) (B). Western blot of Pcdh1 isoform 1 (iso 1, EP78-antibody), isoform 2 (iso 2, EP76-antibody) and the 50kD fragment (EP76-antibody) in lung homogenate of mice exposed to smoke for 1 day (smoke) or air (control) (C). Densitometric quantification of Pcdh1 expression levels of full-length isoform 1 and 2, and the 50kD fragment, relative to β-actin (D). Cig = cigarette; † = time-point at which mice are sacrificed; CS = cigarette smoke.



When comparing the gene-structure of *Pcdh1* to human *PCDH1* (13), we observed a striking homology between the two; with 87% at the nucleotide level and 96% at the amino-acid level (Figure 1A, Supplementary Figure E1). Moreover, the low expression levels of alternative 5'exons (exon m-1b), the gap in exon 4 (13, 16) resulting in deletion of conserved CM2-domains, and the novel exon 5 are also conserved between man and mouse (13). In addition to the high level of conservation we obtained evidence for a third Pcdh1 isoform that

initiates transcription from within exon 2 and contains a CTG start-codon (27) within a conserved KOZAK sequence (Figure 1A). Overexpression of this isoform induced expression of 40-and 50kD PCDHl proteins that were only detected by the antibody generated against the intracellular domain encoded by exon 4 also present in isoform 2 (EP76-antibody). ASMs stained positive only with this specific antibody. These results suggest that ASMs express a Pcdh1 protein containing intracellular, but not extracellular cadherin repeats, consistent with a third isoform. Its function may involve cell signalling rather than cell adhesion, which will be subject of future investigations in human ASM cells. In contrast to ASM cells, this novel isoform was not conclusively expressed in airway epithelial cells, as shown *in vivo* using the combination of approaches employed by us. The 16HBE bronchial epithelial cell-line however, was shown to display a 50kd band on Western blot that could correspond to this novel isoform (13), but it might also represent a posttranslational modification of the full-length isoform 2 protein as has been observed for other protocadherins (28).

Expression of Pcdh1 during mouse development was previously investigated by RNA hybridizations using a probe specific for the three last extracellular domains and the transmembrane domain of Pcdh1 (18). This probe covers *Pcdh1* isoforms containing the extracellular domains (isoform 1 and 2), but does not detect the newly identified intracellular isoform 3. Using this approach *Pcdh1* staining was observed in the parenchyma with a predominant positive staining for blood vessels, but no staining in the bronchi, up to the adult stage (18). This is in contradiction to our results where a clear bronchial epithelial staining, and a lack of blood vessel staining was found by immunohistochemistry using 3 independent antibodies. The smooth muscle layer surrounding the vessels stained positive by the isoform 2/3-specific antibody only, corresponding to expression of Pcdh1 isoform 3. This 3rd isoform would have been missed by the probe used in the RNA hybridization study. The absence of airway epithelial expression of *Pcdh1* during development might be explained by the different developmental stages under scrutiny or differences in the sensitivity of the different techniques used to detect Pcdh1. Clearly, more data with respect to the role of Pchd1 in lung development is needed.

The effect of genetic variation of the AHR susceptibility gene *PCDH1* on its expression and regulation is currently unknown. We hypothesized that gene-variants are associated with alterations in PCDH1 expression, either at the basal level or in response to relevant environmental stimuli, and analyzed the regulation of Pcdh1 expression in mouse

models of experimental asthma, sensitized through the epithelium. In the mild HDM-induced asthma model, whole lung *Pcdh1* mRNA expression remained relatively stable during the active phase (i.e during HDM exposures), but increased in the recovery phase following exposures 4 and 5 (Figure 3). Given the slight increase of *Pcdh1* expression levels in time in the control groups over the course of this HDM-model, we cannot formally distinguish whether these data represent a *repression* of *Pcdh1* expression during the HDM exposures that extends into the first week after these exposures, or an *increase* in *Pcdh1* expression during the recovery phase at later time-points after the cessation of the HDM exposures. Furthermore we can not tell whether the observed differences in *Pcdh1* expression levels arise from changes in epithelial or ASM *Pcdh1*-expression levels, as previously changes in ASM-mass were observed in HDM-exposed mice, as well (24). Nevertheless, the increase in *Pcdh1* expression levels is evident and suggests a role for Pcdh1 in epithelial repair. Epithelial repair consists of several processes, including an immediate response with cell migration to restore the barrier, followed by an interim repair stage and a final phase of proliferation and differentiation (4). Based on our previous observation of an up regulation of PCDH1 expression during mucociliary differentiation of airway epithelial cells (13), we hypothesize that Pcdh1 may play a role in this final phase of epithelial repair after repeated allergen exposures. To get a more detailed understanding of the expression of Pcdh1 during the active phase of allergic asthma development, Pcdh1 protein expression was investigated in our chronic HDM-driven allergic asthma model (19) in which the mice received a double number of HDM exposures and were sacrificed 24h after the last HDM exposure. Interestingly, in this model of ongoing allergic airway inflammation and remodelling, *lower* Pcdh1 isoform 2 or 3 protein levels were found in airway epithelial cells. These epithelial cells were located in airways that were devoid of goblet cell metaplasia. These results suggest that airway exposures to allergen do have impact on epithelial Pcdh1 expression, although these effects are relatively mild and only apparent for Pcdh1 isoform 2 and/or 3.

In addition to chronic HDM exposure, CS-exposure also reduced *Pcdh1* expression levels, as determined in whole lung homogenate. Since loss of *Pcdh1* expression is already observed as early as 6 hours after CS-exposure, *Pcdh1* expression might in fact be regulated directly by CS-exposure as this time-point precedes significant influx of inflammatory cells in the airways. The human chromosomal region 5q31-33, containing *PCDH1*, was previously linked to AHR and asthma in 95 ETS exposed families. In this study, ETS exposure was defined by a significant smoking history of the proband of the asthma family. Associations of

PCDH1 gene-variants with AHR were further investigated in four populations of children exposed to ETS *in utero* or in early life. *PCDH1* gene-variant rs3822357 was associated with AHR in ETS exposed children in one population only (12).

Mice sub chronically exposed to smoke were hyperresponsive to methacholine (trend), which confirms other studies in smoke exposed mice (29) and guinea pigs (30). CS is known to impair the epithelial barrier function, and thereby induces permeability of airway epithelial cells, as evidenced by a decrease in electrical (31) or trans-epithelial (3) resistance. Epithelial cells are interconnected by tight-junctions (TJ) and adherens junctions (AJ). CS delocalizes the TJ adaptor molecule ZO-1 (32), and down-regulates several AJ adhesion molecules, including E-cadherin (33). The resulting loss of TJ-and AJ stability may subsequently lead to loss of barrier function of the epithelium. As *PCDH1* was previously reported to have a role in cell-cell adhesion (34), the loss of PCDH1 expression might contribute to the reduced epithelial barrier function induced by CS-exposure. Since *PCDH1* is an AHR susceptibility gene, we hypothesize that these decreased Pcdh1 levels contribute to a reduced epithelial barrier function or an altered repair process, thereby making subjects more susceptible to environmental insults, possibly leading to inflammation, remodelling and eventually AHR and asthma. Remarkably, in both the HDM-driven asthma model and the CS-exposure model, Pcdh1 expression levels are relatively low at the time when AHR is observed. Future mechanistical studies will assess the contribution of Pcdh1 expression levels to AHR.

In conclusion, our data show that Pcdh1 is strongly conserved between mouse and man. Furthermore, our data are the first to show that Pcdh1 expression is strongly regulated by CS-exposure, while its regulation in HDM-driven models of in experimental allergic asthma is less pronounced, but present. Future studies on the function of Protocadherin-1, using novel knockout and/or transgenic approaches, and its interaction with environmental factors such as HDM and/or CS exposure will provide novel insights into the origins of airway hyperresponsiveness and asthma.

ACKNOWLEDGMENTS

We thank Wim Timens and Marjan Reinders-Luinge (Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, the Netherlands) for advice and assistance with immunohistochemistry, and Klaas A. Sjollema (UMCG Microscopy and Imaging Center (UMIC), University of Groningen, University

Medical Center Groningen, The Netherlands) for assisting with image quantification techniques.

REFERENCES

1. Koppelman GH. (2006) Gene by environment interaction in asthma. Curr Allergy Asthma Rep 6(2): 103-111.
2. Ober C, Yao TC. (2011) The genetics of asthma and allergic disease: A 21st century perspective. Immunol Rev 242(1): 10-30.
3. Xiao C, Puddicombe SM, Field S, Haywood J, Broughton-Head V, et al. (2011) Defective epithelial barrier function in asthma. J Allergy Clin Immunol 128(3): 549-556.
4. Davies DE. (2009) The role of the epithelium in airway remodeling in asthma. Proc Am Thorac Soc 6(8): 678-682.
5. Kicic A, Hallstrand TS, Sutanto EN, Stevens PT, Kobor MS, et al. (2010) Decreased fibronectin production significantly contributes to dysregulated repair of asthmatic epithelium. Am J Respir Crit Care Med 181(9): 889-898.
6. Prefontaine D, Nadigel J, Chouiali F, Audusseau S, Semlali A, et al. (2010) Increased IL-33 expression by epithelial cells in bronchial asthma. J Allergy Clin Immunol 125(3): 752-754.
7. Pichavant M, Charbonnier AS, Taront S, Brichet A, Wallaert B, et al. (2005) Asthmatic bronchial epithelium activated by the proteolytic allergen der p 1 increases selective dendritic cell recruitment. J Allergy Clin Immunol 115(4): 771-778.
8. Hackett TL, Singhera GK, Shaheen F, Hayden P, Jackson GR, et al. (2011) Intrinsic phenotypic differences of asthmatic epithelium and its inflammatory responses to RSV and air pollution. Am J Respir Cell Mol Biol 45(5): 1090-1100.
9. Uller L, Leino M, Bedke N, Sammut D, Green B, et al. (2010) Double-stranded RNA induces disproportionate expression of thymic stromal lymphopoietin versus interferon-beta in bronchial epithelial cells from donors with asthma. Thorax 65(7): 626-632.
10. Ierodiakonou D, Postma DS, Koppelman GH, Boezen HM, Gerritsen J, et al. (2011) E-cadherin gene polymorphisms in asthma patients using inhaled corticosteroids. Eur Respir J 38(5) :1044-1052.
11. Nawijn MC, Hackett TL, Postma DS, van Oosterhout AJ, Heijink IH. (2011) E-cadherin: Gatekeeper of airway mucosa and allergic sensitization. Trends Immunol 32(6): 248-255.
12. Koppelman GH, Meyers DA, Howard TD, Zheng SL, Hawkins GA, et al. (2009) Identification of PCDH1 as a novel susceptibility gene for bronchial hyperresponsiveness. Am J Respir Crit Care Med 180(10): 929-935.
13. Koning H, Sayers I, Stewart CE, de Jong D, Ten Hacken NH, et al. (2012) Characterization of protocadherin-1 expression in primary bronchial epithelial cells: Association with epithelial cell differentiation. FASEB J 26(1): 439-448.
14. Dephoure N, Zhou C, Villen J, Beausoleil SA, Bakalarski CE, et al. (2008) A quantitative atlas of mitotic phosphorylation. Proc Natl Acad Sci U S A 105(31): 10762-10767.
15. Rush J, Moritz A, Lee KA, Guo A, Goss VL, et al. (2005) Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. Nat Biotechnol 23(1): 94-101.
16. Vanhalst K, Kools P, Staes K, van Roy F, Redies C. (2005) Delta-protocadherins: A gene family expressed differentially in the mouse brain. Cell Mol Life Sci 62(11): 1247-1259.
17. Yoshida K, Watanabe M, Kato H, Dutta A, Sugano S. (1999) BH-protocadherin-c, a member of the cadherin superfamily, interacts with protein phosphatase 1 alpha through its intracellular domain. FEBS Lett 460(1): 93-98.
18. Redies C, Heyder J, Kohoutek T, Staes K, Van Roy F. (2008) Expression of protocadherin-1 (Pcdh1) during mouse development. Dev Dyn 237(9): 2496-2505.
19. Post S, Nawijn MC, Hackett TL, Baranowska M, Gras R, et al. (2011) The composition of house dust mite is critical for mucosal barrier dysfunction and allergic sensitisation. Thorax Epub ahead of print.
20. Vlahos R, Bozinovski S, Jones JE, Powell J, Gras J, et al. (2006) Differential protease, innate immunity, and NF-kappaB induction profiles during lung inflammation induced by subchronic cigarette smoke exposure in mice. Am J Physiol Lung Cell Mol Physiol 290(5): L931-45.
21. Andersen CL, Jensen JL, Orntoft TF. (2004) Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64(15): 5245-5250.

22. Heijink IH, Brandenburg SM, Noordhoek JA, Postma DS, Slebos DJ, et al. (2010) Characterisation of cell adhesion in airway epithelial cell types using electric cell-substrate impedance sensing. Eur Respir J 35(4): 894-903.
23. Nawijn MC, Piavaux BJ, Jeurink PV, Gras R, Reinders MA, et al. (2011) Identification of the MHC region as an asthma susceptibility locus in recombinant congenic mice. Am J Respir Cell Mol Biol 45(2): 295-303.
24. Johnson JR, Wiley RE, Fattouh R, Swirski FK, Gajewska BU, et al. (2004) Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling. Am J Respir Crit Care Med 169(3): 378-385.
25. Hammad H, Plantinga M, Deswarte K, Pouliot P, Willart MA, et al. (2010) Inflammatory dendritic cells--not basophils--are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. J Exp Med 207(10): 2097-2111.
26. Botelho FM, Gaschler GJ, Kianpour S, Zavitz CC, Trimble NJ, et al. (2010) Innate immune processes are sufficient for driving cigarette smoke-induced inflammation in mice. Am J Respir Cell Mol Biol 42(4): 394-403.
27. Lock P, Ralph S, Stanley E, Boulet I, Ramsay R, et al. (1991) Two isoforms of murine hck, generated by utilization of alternative translational initiation codons, exhibit different patterns of subcellular localization. Mol Cell Biol 11(9): 4363-4370.
28. Haas IG, Frank M, Veron N, Kemler R. (2005) Presenilin-dependent processing and nuclear function of gamma-protocadherins. J Biol Chem 280(10): 9313-9319.
29. Seymour BW, Schelegle ES, Pinkerton KE, Friebertshauser KE, Peake JL, et al. (2003) Second-hand smoke increases bronchial hyperreactivity and eosinophilia in a murine model of allergic aspergillosis. Clin Dev Immunol 10(1): 35-42.
30. Matsumoto K, Aizawa H, Inoue H, Koto H, Takata S, et al. (1998) Eosinophilic airway inflammation induced by repeated exposure to cigarette smoke. Eur Respir J 12(2): 387-394.
31. Heijink IH, Brandenburg SM, Postma DS, van Oosterhout AJ. (2012) Cigarette smoke impairs airway epithelial barrier function and cell-cell contact recovery. Eur Respir J 39(2): 419-428.
32. Petecchia L, Sabatini F, Varesio L, Camoirano A, Usai C, et al. (2009) Bronchial airway epithelial cell damage following exposure to cigarette smoke includes disassembly of tight junction components mediated by the extracellular signal-regulated kinase 1/2 pathway. Chest 135(6): 1502-1512.
33. Shaykhiev R, Otaki F, Bonsu P, Dang DT, Teater M, et al. (2011) Cigarette smoking reprograms apical junctional complex molecular architecture in the human airway epithelium in vivo. Cell Mol Life Sci 68(5): 877-892.
34. Sano K, Tanihara H, Heimark RL, Obata S, Davidson M, et al. (1993) Protocadherins: A large family of cadherin-related molecules in central nervous system. EMBO J 12(6): 2249-2256.

SUPPLEMENTARY DATA

Supplementary Figure E1: Human PCDH1 and mouse Pcdh1 protein homology

Alignments of human PCDH1 and mouse Pcdh1 protein isoforms 1 and 2 was performed using Clustal W software. Homology of EP78 and EP76 immunizing peptides is depicted in the marked boxes.

Chapter 6

Subcellular localization of protocadherin-1 isoforms and their interaction with SMAD3 in airway epithelial cells

Henk Koning
Uilke Brouwer
Brigitte W.M. Willemse
Grissel Faura Tellez
Peter M. Lackie
Antoon J.M. van Oosterhout
Dirkje S. Postma
Martijn C. Nawijn*
Gerard H. Koppelman*
* = shared last authorship

ABSTRACT

Protocadherin-1 (*PCDH1*) and Mothers against decapentaplegic homolog 3 (*SMAD3*) are novel asthma associated genes. *PCDH1* encodes for two transmembrane molecules with seven extracellular cadherin repeats, and one intracellular isoform containing conserved signalling domains that are shared with one of the transmembrane isoforms. PCDH1 belongs to the δ1 family of non-clustered protocadherins and displays weak adhesion activity. PCDH1 and SMAD3 are proposed to interact on protein level, suggesting that they may act in a novel pathway associated with asthma. We aimed to identify whether PCDH1 is a candidate molecule for intracellular signalling by investigating the subcellular localization of PCDH1 isoforms and their interaction with SMAD3 in 16HBE bronchial epithelial cells. The subcellular localization of PCDH1 protein products was investigated in 16HBE culture supernatant, cytoplasmic, and nuclear/membrane fractions, and by immunofluorescent microscopy using four PCDH1 specific antibodies targeted at both extracellular and intracellular domains. An interaction with SMAD3 was investigated by co-immunoprecipitation in PCDH1 pull-downs using PCDH1 isoform-1 and isoform-2/3 specific antibodies. We identified a novel 120kD soluble PCDH1 protein product (sPCDH1), and a series of novel intracellular 52, 50, 48 and 37kD protein products. Isoform 1 showed a punctuate supra-nuclear localization, while a cytoplasmic filamentous staining pattern was detected for isoform-2/3 that concentrated around the nucleus. Furthermore, an interaction of both PCDH1 isoforms 1 and 2 with SMAD3 was identified by SMAD3 co-immunoprecipitation in PCDH1 pull-downs and *vice versa*, at endogenous levels in 16HBE cells. We conclude that PCDH1 may contribute to signalling by its soluble extracellular sPCDH1 isoform and via intracellular protein products that reside at the nuclear membrane. Furthermore, by identifying the interaction of both PCDH1 isoforms with SMAD3 we physically connect two asthma associated genes to act in a shared pathway.

INTRODUCTION

Asthma is a chronic disease of the airways, affecting up to 300 million individuals world-wide (1). Asthma is a complex disease, characterized by gene-gene and gene-environment interactions (2). Recent genetic studies have led to the discovery of novel, replicated asthma loci such as *ORMDL3/GSMDL*, *IL1RL1/IL18R1*, *TSLP/WDR36, IL-33*, and *SMAD3* identified by genome wide association studies (3), and *ADAM33* (4), *NPSR1* (5) and *PCDH1* (6) by positional cloning. Although these discoveries likely identify genes associated with important mechanisms in asthma, it has been difficult to assign these novel genes to distinct pathways, with notable exceptions being IL33 – IL1RL1 signalling (7), and the NPSR1 - Tenascin C interactions (8). Two of these novel asthma genes, Protocadherin-1 (*PCDH1*) and Mothers against decapentaplegic homolog 3 (*SMAD3*), were proposed to interact on protein level based on yeast-2-hybrid screens (9, 10). This suggests that PCDH1 and SMAD3 might act in a third pathway identified by unbiased genetic screens in asthma.

SMAD3 is widely expressed in many cell types including epithelial, mesenchymal and immune cells (11, 12). Upon TGFβ or Activin (Act) activation (13), the constitutive active type II TGFβ or Act - Receptor kinases form a complex with and subsequently phosphorylate the type I receptors, such as TGFβ Receptor I (TGFβRI) or Activin Like Kinase (ALK) receptors. Subsequently, these type 1 receptors phosphorylate SMAD3 C- terminally. Phosphorylated SMAD3 binds to SMAD4, translocates to the nucleus, and induces gene-transcription (14).

PCDH1 belongs to the δ1-protocadherin subfamily of the cadherin superfamily of adhesion molecules, and is expressed in the airway epithelium (6) where its levels are upregulated during mucociliary differentation (15). *PCDH1* encodes for two full-length high molecular weight isoforms (isoform 1 of 150kD, isoform 2 of 170kD), both containing seven extracellular cadherin repeats (ECs), and a third isoform (isoform 3 of approximately 50kD) that lacks 5 or all ECs and thus mostly consists of intracellular domains (15) (Figure 1). The intracellular domains of isoforms 2 and 3 are characterized by the presence of three conserved signalling motifs CM1-3. Whereas the putative interaction partners of CM1 and 2 are unknown, CM3 is the binding domain for protein phosphatase 1 alpha (PP1α) (16), a phophatase implicated in lung development (17).

PCDH1 may function as an adhesion molecule, but displays only weak adhesion activity (18). Despite its proposed interactions with downstream molecules like PP1α and SMAD3 the possibility that PCDH1 performs other functions like intracellular signalling has not been investigated. Adhesion molecules may perform signalling functions, by (a) clustering with signalling receptors, (b) binding cytoskeleton organizing molecules, (c) interacting with intracellular signal transduction proteins, or (d) by translocating to the nucleus to affect gene-transcription (reviewed in (19)). Extracellular domains of (proto)cadherins may be shed by metalloproteases, followed by cleavage of the intracellular domain by presenilins, as was described for E-cadherin (20) and γ-Protocadherins (21). In addition, transcriptional or translational regulation may lead to the expression of isoforms that only encode extracellular domains (as was shown for PCDH15, (22)) or lack extracellular domains (as was reported for PCDH1 and PCDH15 (15, 22)). Altogether, the subcellular localization of PCDH1 isoforms may provide a first clue into its potential signalling functions. We hypothesize that PCDH1 can contribute to intracellular signalling. Therefore we determined the subcellular localization of PCDH1 isoforms, and their interaction with a known downstream signalling molecule, SMAD3, in bronchial epithelial cells.

MATERIAL AND METHODS

Cell-culture

The human bronchial epithelial cell line 16HBE14o- (16HBE) was cultured in Eagle minimum essential medium (EMEM) supplemented with 10% fetal calf serum (FCS; Biowhittaker, Verviers, Belgium) on collagen (PureCol, Inamed, Santa Barbara, California, USA) and bovine serum albumin (A3311-100G, Sigma-Aldrich, Zwijndrecht, The Netherlands) coated T25-flasks as described previously (23). In order to generate a time-range with increasing confluency of 16HBE cells, $7*10^5$ cells were seeded in T25-flasks at day 0, and harvested after 24h (day 1), 72h (day 3) and 120h (day 5) in duplicate. In total three independent replicate experiments were performed.

Measurements of PCDH1 in 16HBE culture supernatants

16HBE supernatants were concentrated with Amicon Ultra-2 Centrifugal Filter Units with Ultracel-10kD membrane (Millipore BV, Amsterdam Zuidoost, The Netherlands), according to the manufacturers' instructions. In brief, 2 ml of supernatant of confluent 16HBE

cells cultured without serum for 24 or 48h in T25-flasks, or control EMEM-medium, was applied to the insert of the 10kD cutoff Amicon column and centrifuged at 4000g for 20 min. The concentrated supernatant was recovered and centrifuged in the inverted column at 1000g for 1 min. The volume of the concentrate was determined, followed by boiling for 5min with the corresponding volume of 5x Leammli buffer (0.3M Tris-HCl pH 6.8, 50% glycerol, 25% β-mercaptoethanol, 10% sodium dodecyl sulphate (SDS), 0.2% bromophenol blue), and stored at -20°C until further use. This experiment was repeated three times.

Preparation of nuclear/membrane extracts from 16HBE epithelial cells

Nuclear/membrane extracts were obtained according to the protocol of Schreiber and co-workers (24). All steps were performed on ice or at 4°C. In brief, 16HBE bronchial epithelial cells were washed with PBS and scraped in ice-cold PBS with protease inhibitor cocktail (1:500, P8340-1ML, Sigma-Aldrich). The cell-pellet was carefully resuspended and incubated for 15 min in 400 µl ice-cold buffer A (10mM HEPES pH 7.9; 10mM KCl; 0.1mM EDTA; 0.1mM EGTA, 1mM DTT, Protease inhibitor cocktail (1:500)). Then Nonidet P-40 (NP40, Sigma Aldrich) was added and incubated for 10 sec to swell the cells followed by collection of the cytoplasmic fraction. After centrifugation at 13000rpm (17982g) for 30 sec the pellet containing the nuclear fraction, precipitated membranes and DNA was washed twice with buffer A to remove remaining cytoplasmic contaminants. Nuclear proteins were extracted by a 15 min incubation with 50 µl ice-cold buffer C (20mM HEPES pH 7.9; 0.4M NaCl; 1mM EDTA; 1mM EGTA, 1mM DTT, Protease inhibitor cocktail (1:500)), followed by centrifugation for 5 min at 13000rpm. The remaining cell-pellet or insoluble fraction was discarded. Protein concentration of extracts was determined using the Micro-BCA kit (Pierce, Thermo Fisher Scientific; Perbio Science BV, Etten-Leur, The Netherlands). Equal protein amounts (5-10 µg) were loaded on 10% acrylamide SDS-page gels for western blotting. Purity of nuclear and cytoplasmic fractions was confirmed by staining for HDAC2 (nuclear protein, 3602-100, BioVision, Mountain View, USA) and α-tubulin (cytoplasmic protein, T6199-200UL, Sigma-Aldrich, Zwijndrecht, The Netherlands) in both fractions. PCDH1 levels in fractions were compared to levels of E-cadherin, an adherens junction protein (H108, Santa-Cruz Biotechnology, Heidelberg, Germany). Potential plasma membrane contamination was assessed by staining for the presence of the Anti-alpha 1 Sodium Potassium ATPase (Plasma membrane marker, ab7671, Abcam, Cambridge, United Kingdom).

Western blot detection of PCDH1

To detect the extracellular domains of PCDH1 by western blotting two antibodies were used: the extracellular cadherin repeat 1 (EC1)-domain antibody (amino-acid 62-170, H00005097-M01, Abnova, Taiwan) and the EC7-domain antibody (EP209 polyclonal antibody; immunizing peptide DIAGPEYERSKQRG+C, (6)). To detect the intracellular domains of PCDH1 by western blotting or immunoprecipitation two polyclonal antibodies were used: the isoform-1 antibody (EP78, immunizing peptide QPFQLSTPQPLPHPYH) and the isoform-2/3 antibody (EP76, immunizing peptide SPSPPEDRNTKTAPV+C) (15). Location of the peptides recognized by the individual antibodies is schematically depicted in Figure 1. PCDH1 proteins present in concentrated culture supernatants and nuclear and cytoplasmic fractions were detected by western blotting as described previously (15). In brief, protein samples were separated on 10% acrylamide SDS-page gels (Bio-Rad Laboratories, Veenendaal, The Netherlands), transferred to nitrocellulose membranes for PCDH1 detection and visualized by EC1-domain, EC7-domain, isoform-1 and-2/3 antibodies, with Goat anti-Rabbit and Rabbit-anti-Goat-Horseradish Peroxidase (HRP, Daco, Heverlee, Belgium) detection antibodies. Blots were incubated with Luminol, and subsequently analysed using Fuji Medical X-Ray films (Fuji Photo Film Gmbh, Germany).

Immunofluoresence

16HBE bronchial epithelial cells were seeded on coverslips ($5*10^4$ cells per coverslip), grown to full confluency and fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in PBS for 20 min and afterwards with 50nM ammonium chloride in PBS for 5 min to quench free aldehyde groups (PCDH1 isoforms 1 and 2 staining), or in ice-cold methanol (Claudin-3, Life Technologies Ltd, Paisley, United Kingdom) for 20 min. Next, cells were incubated with blocking buffer (10% normal goat serum or 1% BSA in PBS) for 20 minutes. Primary antibodies (PCDH1 isoform-1 ab (1:50), isoform-2/3 ab (1:20), EC7-domain ab (1:200) or Claudin-3 (1:50) diluted in PBS with 0.1% BSA) or PBS with 0.1% BSA (negative control) were applied for 60 min. Finally, goat anti-rabbit Alexa Fluor 488 conjugate (green fluorescence, Life Technologies Ltd) was used as a secondary antibody to visualize PCDH1 or Claudin-3 immunoreactivity, while nuclei were counterstained with Hoechst 33342 (blue fluorescence, Molecular Probes, Life Technologies Ltd). Fluorescence staining was detected by Confocal Microscopy using a Leica SP5 Confocal microscope.

Immunoprecipitation (IP) of PCDH1 and SMAD3 proteins

Confluent 16HBE cells (T25-flask) were scraped in ice-cold Mammalian Protein Extraction Reagent buffer (M-PER, Pierce, Thermo Fisher Scientific) with protease inhibitor cocktail (1:250; Sigma-Aldrich), and lysed at 4°C using an orbital shaker. The lysate was pre-centrifuged to remove insoluble proteins and pre-cleared by incubating with Protein-G agarose beads (Pierce, Thermo Fisher Scientific) to reduce non-specific binding of proteins to fresh beads at a later stage. Next, 1µg of isoform-specific PCDH1 antibody (isoform-1 or 2/3) or 2 µl of SMAD3 antibody (04-1035, Millipore) was added per 100 µl lysate, and incubated overnight at 4°C to bind PCDH1 or SMAD3 proteins. The immune complexes were precipitated after 3h incubation with fresh Protein-G agarose beads, and washed thoroughly with PBS. As a control, lysate was incubated at the same time without PCDH1 or SMAD3 antibody followed by incubation with fresh Protein-G agarose beads. The bound immune complexes or control beads were boiled in 5x Laemmli buffer to elute proteins and used for western blotting purposes. Immunoprecipitated proteins were separated on SDS-PAGE gels and blotted to nitrocellulose membranes for PCDH1 detection as described before, or transferred to PDVF membranes for SMAD3 detection using the SMAD3 detection antibody C67H9 (Cell-Signalling Technology, Bioké, Leiden, The Netherlands). Antibodies used for detection of immunoprecipitated proteins of approximately 50kD were first biotin-labelled using the EZ-link Sulfo-NHS-LC-Biotin kit (Thermo Fisher Scientific), followed by detection using Streptavidin-HRP (R&D Systems Europe Ltd, Oxon, United Kingdom) as secondary step, in order to prevent interference of the 50kD heavy-chain IgG molecules.

Quantification of protein levels

Protein intensity levels (*I*) were quantified by densitometric analysis using Quantity One 4.6.2 software (Bio-Rad). α-Tubulin was used as a marker for the cytoplasmic fraction, while HDAC2 was used as a marker for the nuclear fraction. The relative contamination of cytoplasmic (*Cyto*) proteins in the nuclear (*Nucl*) fraction (*a*), and nuclear proteins in the cytoplasmic fraction (*b*) was calculated as follows:

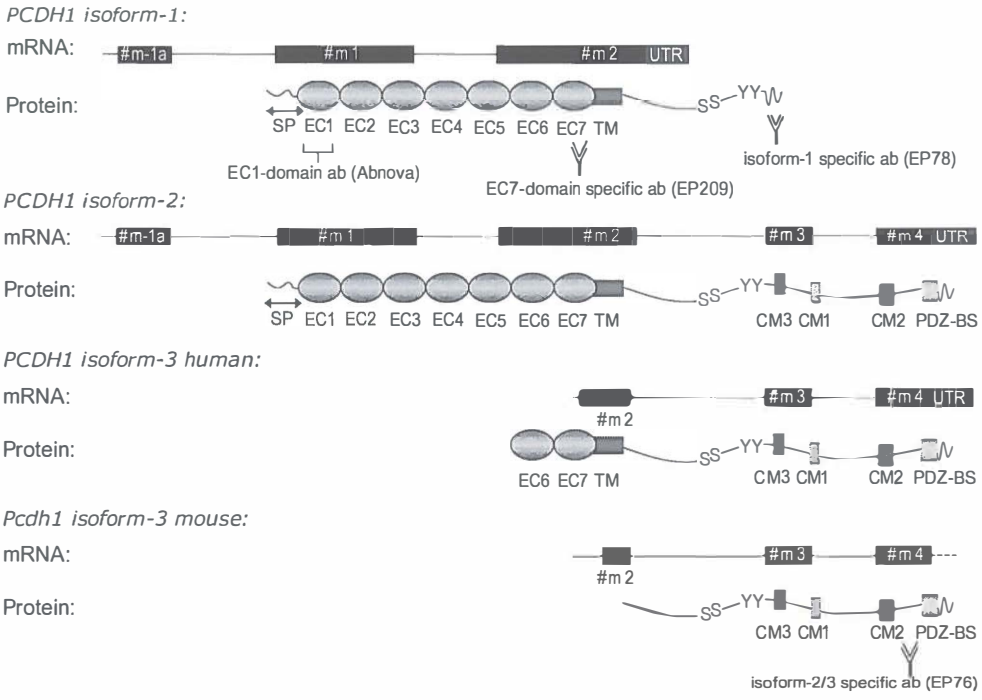
$$a(\%) = \frac{I_{\alpha-tubulin} Nucl}{(I_{\alpha-tubulin} Cyto + I_{\alpha-tubulin} Nucl)} \times 100; b(\%) = \frac{I_{HDAC2} Cyto}{(I_{HDAC2} Nucl + I_{HDAC2} Cyto)} \times 100$$

RESULTS

Detection of soluble PCDH1 in 16HBE tissue culture medium

We previously identified PCDH1 proteins of 150kD (isoform 1), 170kD (isoform 2) and ~50kD (isoform 2 or 3) PCDH1 protein products in whole cell-lysate of primary bronchial epithelial cells and 16HBE bronchial epithelial cells (15). Here, we aimed to identify the presence of PCDH1 proteins in tissue culture supernatants, using EC1 and EC7-domain antibodies (abs) (directed against extracellular PCDH1 domains) and isoform 1 and 2/3 abs (directed against intracellular PCDH1 domains) respectively (see Figure 1).

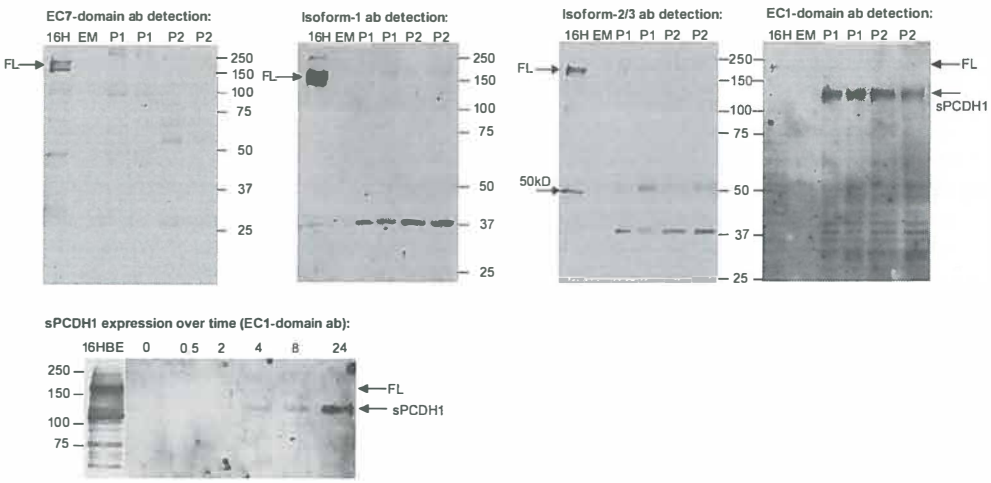
Figure 1: PCDH1 isoforms and epitopes of detection antibodies
Schematic overview of both mRNA and protein structures of PCDH1 isoforms 1, 2 and 3. The potentially phosphorylated Serine (S) and Tyrosine (Y) residues are located in the constant shared region of all isoforms. Conserved motifs (CM) encoded by exon 3 and 4 are present only in isoforms 2 and 3. Epitopes of PCDH1 antibodies are depicted. SP = signal peptide, EC = extracellular cadherin repeat, TM = transmembrane domain, PDZ-BS = PDZ-domain binding site.



37kD fragments (both isoform-1 and 2/3 abs) and a 50kD fragment (isoform-2/3 ab) were detected in tissue culture medium of 16HBE cells (Figure 2). The 37kD fragments were confirmed to be specific after peptide pre-incubation of the abs, while the 50kD fragment was non-specific (results not shown). Interestingly, a clear 120kD protein product was detected using the antibody directed against the EC1-domain of PCDH1, that appeared as early as 4 hours after incubation of confluent 16HBE epithelial cells with fresh serum free tissue culture medium. No full-length PCDH1 isoforms were detected in tissue culture medium of 16HBE cells (Figure 2). These results may suggest that PCDH1 is actively being shed, resulting in the presence of a soluble 120kD PCDH1 isoform (sPCDH1) that contains extracellular but not intracellular domains. Furthermore it may suggest that PCDH1 is secreted, resulting in 37kD proteins only containing intracellular domains of isoform 1 or isoforms 2/3.

Figure 2: Detection of PCDH1 isoforms in 16HBE culture medium

Concentrated 16HBE supernatants were screened for the presence of PCDH1 protein products using EC7-domain, isoform-1, isoform-2/3 and EC-1 domain antibodies (upper panels). The appearance of sPCDH1 was determined in a time-range using the EC1-domain antibody (lower panel). Equal volumes of concentrated supernatant were loaded in each lane. 16H = 16HBE cell-lysate, EM = EMEM control medium, P1 = Passage 1, P2 = Passage 2, FL = full-length, sPCDH1 = soluble PCDH1, 50kD = 50kD PCDH1 protein product.

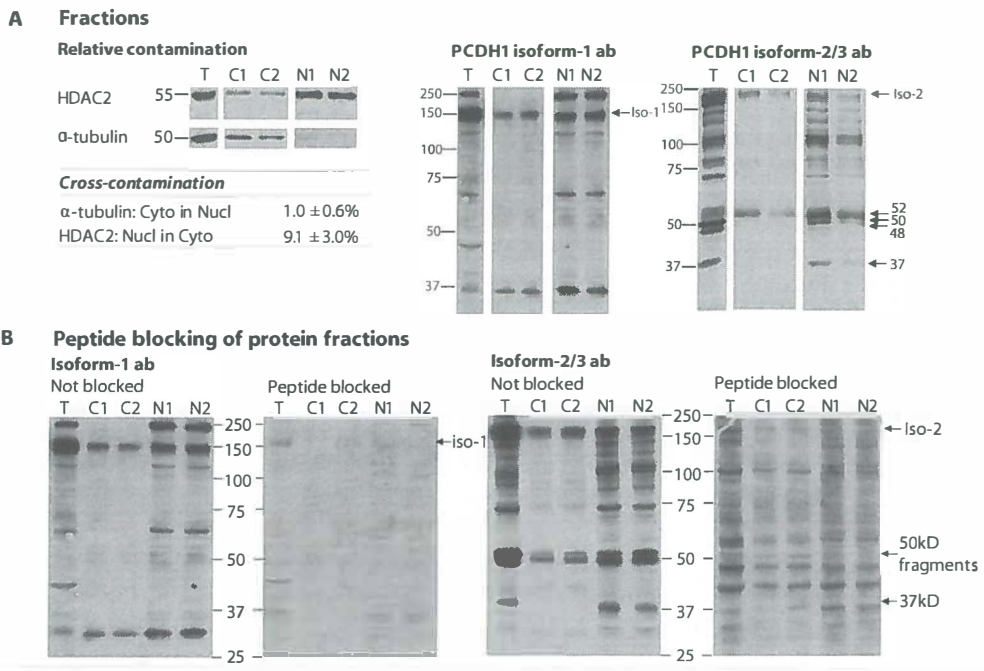


Localization of PCDH1 isoforms in subcellular fractions

We next investigated the relative presence of PCDH1 isoforms in cytoplasmic and nuclear/membrane fractions of confluent 16HBE epithelial cells. We were able to efficiently separate the cytoplasmic fraction from the nuclear/membrane fractions as evidenced by the detection of 1.0% of the cytoplasmic protein α-tubulin in the nuclear/membrane fraction, and of 9.1% of the nuclear protein HDAC2 in the cytoplasmic fraction (Figure 3A). This implies that the nuclear/membrane fraction consists of approximately 99% nuclear/membrane proteins with 1% cytoplasmic protein contamination, while the cytoplasmic fraction consists of 90.9% cytoplasmic proteins with 9.1% nuclear protein contamination. We detected full-length PCDH1 isoforms 1 and 2 proteins both in nuclear/membrane and cytoplasmic fractions (Figure 3). In addition, using the isoform 2/3-antibody we detected multiple low molecular weight PCDH1 protein bands (50, 48 and 37kD) that were exclusively present in the nuclear/membrane fraction, and one low molecular weight PCDH1 band (52 kD) that was present in both nuclear and cytoplasmic fractions (Figure 3A). The specificity of the Ab staining for these fragments was confirmed by blocking of the signal by pre-incubation with immunizing peptides (Figure 3B).

Previously we observed an increase in expression levels of the full-length 150kD isoform 1 and 170kD isoform 2 PCDH1 proteins with epithelial differentiation (15). Therefore, we investigated whether the expression of the 37-52kD PCDH1 protein products

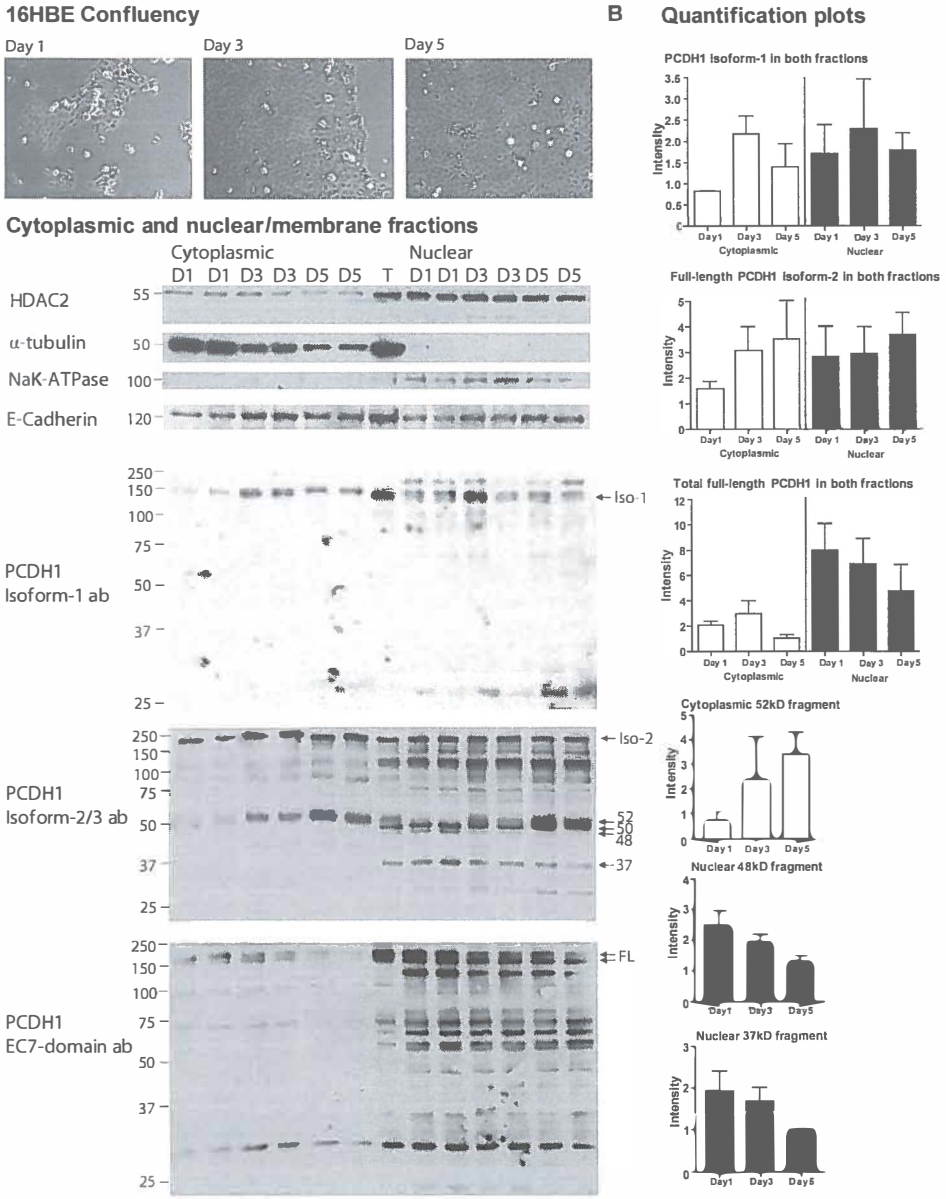
Figure 3: Detection of PCDH1 isoforms in 16HBE subcellular fractions
Cytoplasmic and nuclear/membrane fractions were screened for the presence of PCDH1 protein products using isoform-1 and isoform-2/3 antibodies. The relative contamination of cytoplasmic and nuclear proteins in the corresponding fractions was calculated using HDAC2 (nuclear marker) and α-tubulin (cytoplasmic marker) expression levels. Equal protein amounts were loaded in each lane (A). The specificity of the detected PCDH1 protein products was determined by blocking experiments by pre-incubation of isoform-1 and isoform-2/3 antibodies with immunizing peptides (B). T = total 16HBE cell lysate, C1 = cytoplasmic fraction 1, C2 = cytoplasmic fraction duplicate, N1 = nuclear fraction 1, N2 = nuclear fraction duplicate, Iso-1 = full-length isoform-1, Iso-2 = full-length isoform-2.



and the 150kD isoform 1 and 170kD isoform 2 PCDH1 proteins change over time with increasing confluence of 16HBE cells, since this bronchial epithelial cell line is known to increase cell-cell contacts during growth and forms adherens and tight-junctions (23). To this end, we compared subcellular distribution of PCDH1 proteins between 16HBE at low confluence (20% confluent; day 1), at sub-confluence (80% confluent; day 3) and full confluence (day 5) (Figure 4A). The 150kD isoform 1 PCDH1 protein was present in both cytoplasmic and nuclear/membrane fractions at low levels, and did not consistently change over time (Figure 4). In contrast, levels of full-length isoform 2 and the low molecular weight band at 52kD increased in the cytoplasmic fraction towards day 5 (Figure 4B). In the nuclear/membrane fraction we observed a stable level of full-length isoform 2 PCDH1 protein. The low-molecular weight bands in the nuclear fraction display variable levels of PCDH1 proteins, with the exception of 48 and 37kD fragment bands that decrease from day 1 towards day 5 (Figure 4B). Remarkably, total full-length PCDH1 protein levels seem to

Figure 4: Detection of PCDH1 isoforms in 16HBE subcellular fractions over time
The change in expression of full-length 150kD isoform 1 (isoform-1 ab, n=2), full-length 170kD isoform 2, and 37-52kD protein products (isoform-2/3 ab, n=3) and both full-length PCDH1 proteins (EC7-domain ab, n=3), in cytoplasmic and nuclear/membrane fractions

were determined with increasing confluence, with low confluence at day 1 (D1), subconfluence at day 3 (D3), and high confluence at day 5 (D5). Representative images of 16HBE confluency are shown. HDAC2, α-tubulin, NaK-ATPase, and E-cadherin were used as nuclear, cytoplasmic, membrane and adherens-junction markers respectively. Equal protein amounts were loaded in each lane (A). Expression levels of full-length 150kD, 170kD, 37-52 kD and total full-length PCDH1 protein products were quantified in both fractions (B). T = total 16HBE cell-lysate, Iso-1 = isoform 1, Iso-2 = Isoform 2, FL = full length.

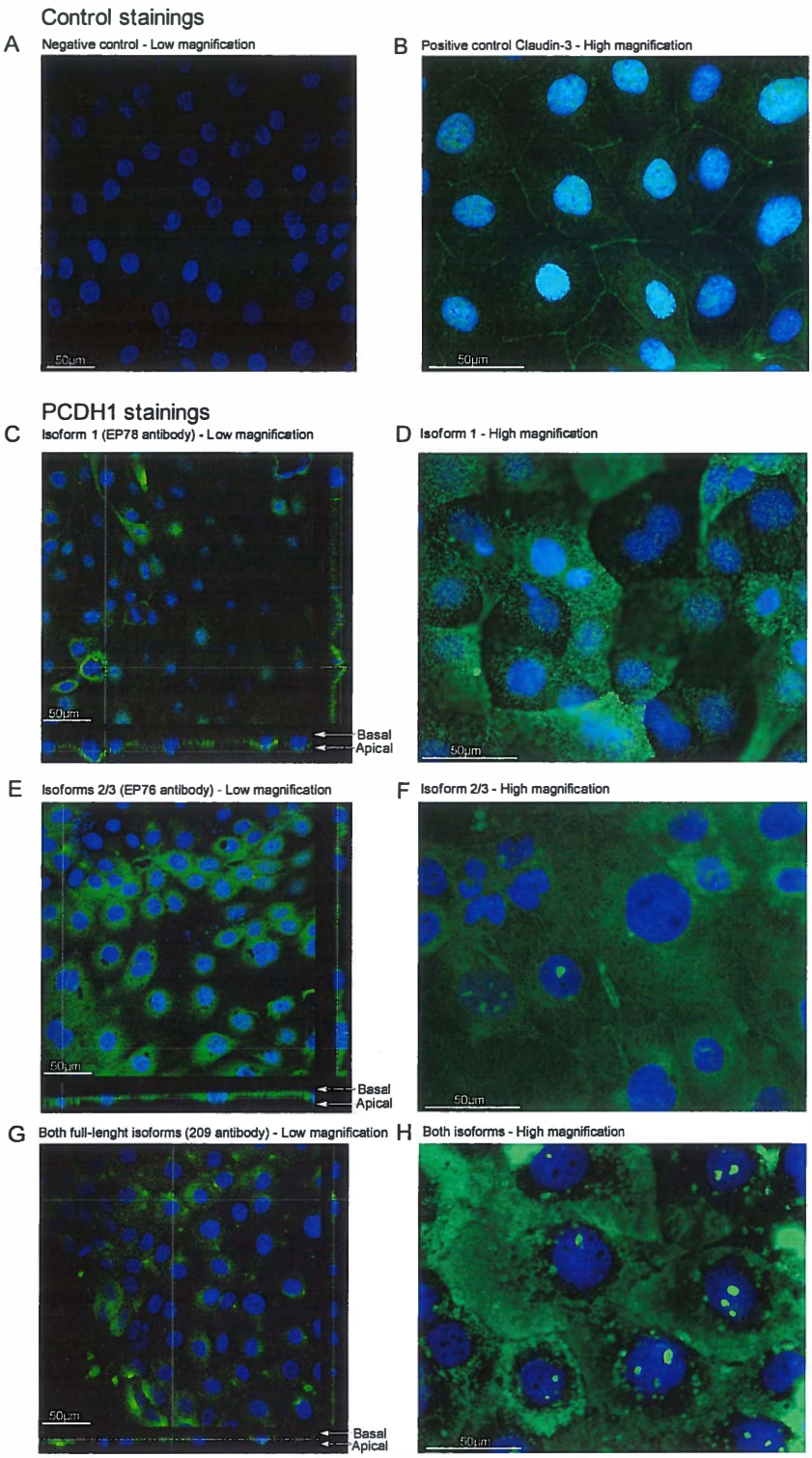


decrease over time both in nuclear and cytoplasmic fractions as evidenced by immunoblotting using the EC-7 domain antibody (Figure 4A and B).

Immunofluorescence localization of PCDH1 isoforms

In order to determine the exact subcellular localization of the PCDH1 isoforms, we next investigated their localization by immunofluorescence staining, using confluent 16HBE monolayers. The negative control showed no cellular staining (Figure 5A). PCDH1 Isoform 1 (isoform-1 ab) showed a punctuate cytoplasmic immunostaining distributed over the entire cell (xy-plane), that is not localized at the plasma-membrane. Interestingly, an orthogonal cross-section of the16HBE monolayer revealed a supra-nuclear staining, suggesting that isoform 1 localizes to the apical part of the cell (xz-plane) (Figure 5C, D). PCDH1 isoforms 2 and/or 3 (isoform-2/3 ab) showed an even distribution over the cell, with no apparent supra-nuclear localization. A higher magnification revealed a dense filamentous staining, with a concentration of signal at the nuclear membrane (Figure 5E, F). Staining of both full-length PCDH1 isoforms 1 and 2 (EC7-domain ab) revealed a combination of punctuate and filamentous staining patterns (Figure 5G, H). The localization of both isoforms was not coincident with Claudin-3, a tight-junction molecule that localized to cell junctions at the plasma-membrane (Figure 5B). Clearly, isoform 1 and 2/3 show a distinct localization pattern in 16HBE bronchial epithelial cells.

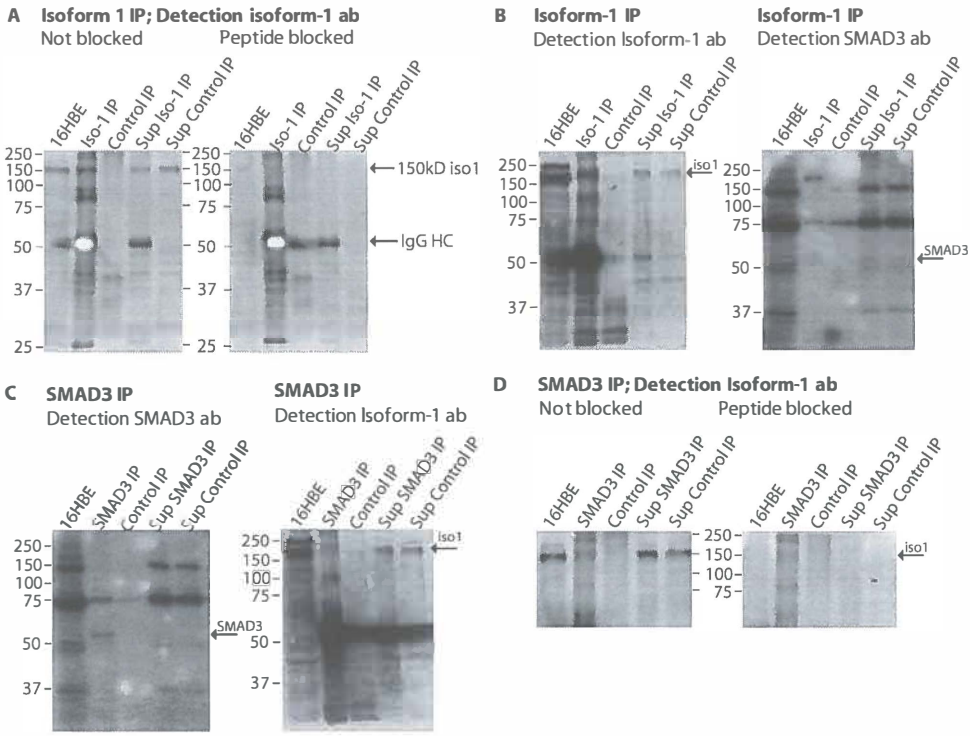
Figure 5: Localization of PCDH1 isoforms in confluent 16HBE monolayers
Immunofluorescent staining of 16HBE epithelial cells without primary antibody (negative control) or with Claudin-3 antibody (positive control), followed by detection with secondary goat anti-rabbit Alexa Fluor 488 conjugate antibody (A, B). Immunofluorescent stainings of 16HBE epithelial cells for PCDH1 isoform 1 (C, D), isoform 2/3 (E, F) or both full-length isoforms (EC7-antibody) (G, H), followed by detection with secondary goat anti-rabbit Alexa Fluor 488 conjugate antibody. Shown are images taken at low magnification including x-y, x-z and y-z sections (left panels (C, E, G); the x-z and y-z orthoganol sections for the lines indicated on the x-y sections are shown on the left and bottom of each image), or at high magnification in x-y plane (right figures (D, F, H)). Scale-bar represents a size of 50μm.


Control stainings
A
Negative control - Low magnification
50µm
B
Positive control Claudin-3 - High magnification
50µm
PCDH1 stainings
C
Isoform 1 (EP78 antibody) - Low magnification
50µm
Basal
Apical
D
Isoform 1 - High magnification
50µm
E
Isoforms 2/3 (EP76 antibody) - Low magnification
50µm
Basal
Apical
F
Isoform 2/3 - High magnification
50µm
G
Both full-lenght isoforms (209 antibody) - Low magnification
50µm
Basal
Apical
H
Both isoforms - High magnification
50µm

PCDH1 isoforms interact with SMAD3

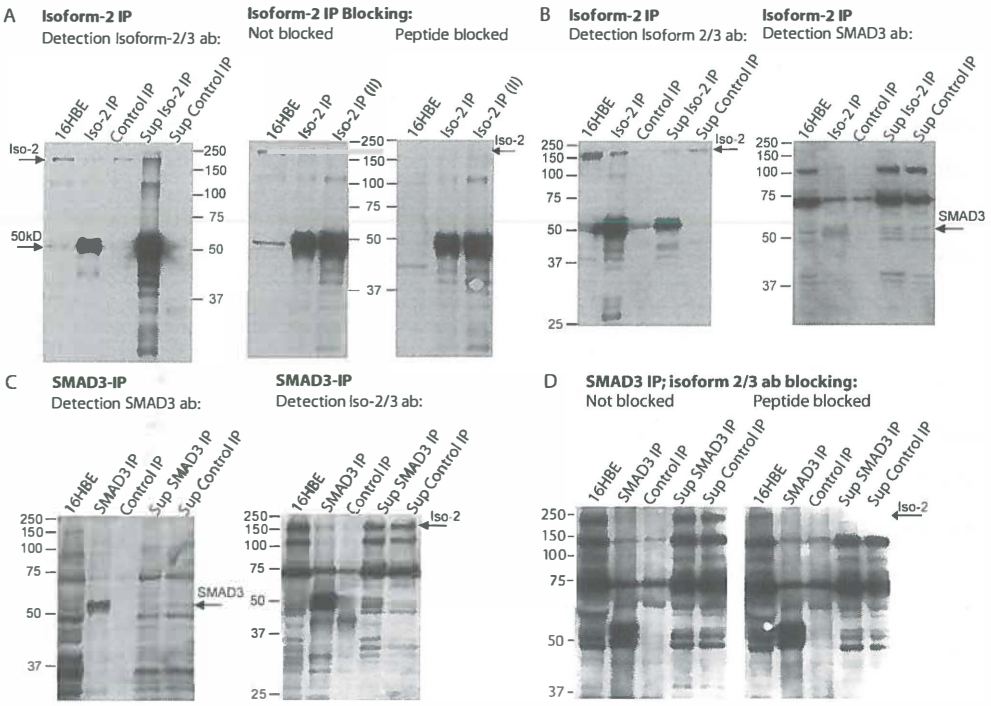
SMAD3 is a novel asthma gene encoding a signal transduction protein that is localized both to the cytosol and nucleus of epithelial cells (25). Previously, SMAD3 was identified as a PCDH1 interacting protein using overexpression yeast-2-hybrid (Y2H) systems (9, 10). To determine whether PCDH1 interacts with SMAD3 at endogenous levels in airway epithelial cells, we performed immunoprecipitation (IP) of PCDH1 proteins using isoform-1 and isoform-2/3 abs, followed by detection of PCDH1 and SMAD3. Detection of a specific 150kD PCDH1 isoform 1 protein band indicated that we were able to IP the endogenous protein from the 16HBE cell lysates (Figure 6A). Next we immunoprecipitated PCDH1 isoform 1, and determined whether SMAD3 (molecular weight of ~54kD) does co-IP with PCDH1. Indeed, we detected low levels of SMAD3 in the PCDH1 isoform 1 IP (Figure 6B). Importantly, biotinylated SMAD3 specific antibody did not detect 50kD heavy-chain IgG molecules (Supplementary Figure E1). To further validate the interaction between PCDH1 isoform 1 and SMAD3, we also immunoprecipitated SMAD3 and tested for co-IP of PCDH1. SMAD3 was successfully immunoprecipitated from 16HBE cell lysates (Figure 6C). Using the isoform-1 specific antibody, we detected the PCDH1 isoform 1 protein of 150kD in the SMAD3 immunoprecipitate (Figure 6C), which was blockable after pre-incubation of the isoform-1 antibody with immunizing peptide (Figure 6D). These results suggest that PCDH1 protein isoform 1 interacts with SMAD3 at endogenous levels in 16HBE cells.

Figure 6: Detection of SMAD3 after immunoprecipitation of PCDH1 isoform 1
Immunoprecipitation of PCDH1 isoform 1 protein from 16HBE cell-lysate using the isoform 1 antibody. Specificity of isoform 1 detection was indicated by pre-incubation of the isoform 1 antibody with immunizing peptide. The arrow depicts the location of the isoform 1 protein band (A). Immunoprecipitation of isoform 1 and; detection using the isoform 1 antibody (left panel); detection of SMAD3 using the SMAD3 antibody (right panel) (B). Immunoprecipitation of SMAD3 using the SMAD3 IP antibody and; detection with SMAD3 detection antibody (left panel); detection of PCDH1 using the isoform-1 antibody (right panel) (C). Specificity of PCDH1 isoform 1 detection after SMAD3 immunoprecipitation was confirmed by blocking experiments of isoform 1 antibody with immunizing peptides (D). Control-IP = IP procedure performed without primary antibody; HC = heavy chain; IP = immunoprecipitation; sup = supernatant; iso-1 = isoform 1; ab = antibody.



In order to determine whether PCDH1 isoform 2 also interacts with SMAD3, we additionally performed IP-experiments using the isoform-2/3 ab. Detection of specific full-length PCDH1 isoform 2 protein band indicated that we were able to successfully IP the endogenous protein from 16HBE cell lysates (Figure 7A). Next, we tested whether SMAD3 is co-immunoprecipitated with PCDH1 isoform 2 from 16HBE cells. As shown in Figure 7B, we clearly identified a SMAD3 band in the PCDH1 isoform 2 IP. In order to validate the interaction between SMAD3 and PCDH1 isoform 2, we also performed SMAD3 IPs, and tested whether PCDH1 isoform 2 would co-IP from the 16HBE cell lysates with SMAD3. We could clearly IP SMAD3 from 16HBE cells (Figure 7C). As expected, PCDH1 isoform 2 could readily be detected in the SMAD3 IP (Figure 7C). Specificity of isoform 2 bands was confirmed by peptide pre-incubation of the biotin-labelled isoform-2/3 ab (Figure 7D). These results confirm the protein-protein interaction of PCDH1 isoform 2 with SMAD3 at endogenous levels in 16HBE cells.

Figure 7: Detection of SMAD3 after immunoprecipitation of PCDH1 isoform 2
Immunoprecipitation of full-length PCDH1 isoform 2 protein from 16HBE cell-lysate using the isoform-2/3 antibody, and detection with both isoform-2/3 (left panel) and EC7-domain (middle panel) antibodies. Specificity of isoform 2 detection was proven by pre-incubation of the isoform-2/3 antibody with immunizing peptide (right panel). The arrow depicts the location of the isoform 2 protein band (A). Immunoprecipitation of isoform 2 and; detection of isoform 2 using isoform-2/3 antibody (left panel); detection of SMAD3 using the SMAD3 detection antibody (right panel) (B). Immunoprecipitation of SMAD3 using the SMAD3 IP antibody and; detection with SMAD3 detection antibody (left panel); detection of PCDH1 isoform 2 (iso-2) using the isoform-2/3 antibody (right panel) (C). Specificity of PCDH1 isoform 2 detection after SMAD3 immunoprecipitation was confirmed by blocking experiments of isoform-2/3 antibody with immunizing peptides (D). IP = immunoprecipitation; sup = supernatant; iso-2 = isoform 2; ab = antibody.



DISCUSSION

PCDH1 is a bronchial hyperresponsiveness (BHR) and asthma gene of unknown function, and is associated with bronchial epithelial cell differentiation (15). Here, we investigated whether PCDH1 can participate in intracellular signalling, by determining the subcellular localization of PCDH1 isoforms and their interaction with SMAD3. Our main results include (i) the identification of a novel soluble PCDH1 protein product (sPCDH1) of 120kD in the culture supernatant that contains extracellular cadherin repeats, and novel secreted 37kD protein products containing intracellular domains; (ii) the identification of intracellular isoform 2 or 3 PCDH1 protein species of 52, 50, 48 and 37 kD containing conserved motifs, that may reside in the cytoplasmic (52kD) or nuclear/membrane compartments (52, 50, 48 and 37 kD); and (iii) the detection of a cytoplasmic supra-nuclear localization of full-length isoform 1, and a filamentous staining pattern for isoforms 2 and/or 3 that concentrated around the nucleus. Finally (iv), we confirmed an interaction of both PCDH1 isoforms 1 and 2 with SMAD3 at endogenous levels in airway epithelial cells, thus physically linking two novel asthma genes, implicating that these act in a single pathway.

This is the first report describing the existence of a PCDH1 protein product that is free in solution and not anchored to the cell membrane. We detected this 120kD sPCDH1 product, containing EC-domains in cell-free 16HBE culture supernatants. The 120kD sPCDH1 may arise from shedding of the full-length isoforms from the plasma membrane by the proteolytic activity of metalloproteases such as ADAMs or MMPs, as this mechanism has been observed previously with ADAM10 for E-cadherin (20), γ-protocadherins (26), and Protocadherin-12 (PCDH12, (27)). Alternatively, the 120kD sPCDH1 may be translated from a novel mRNA transcript, as was previously observed for PCDH15 (22). However, in our own RACE experiments in bronchial epithelial cells from asthma patients, we have not found evidence for the presence of such an mRNA transcript for PCDH1 (see Chapter 4). The function of the sPCDH1 fragment is currently unknown, although two possibilities can be envisioned: (i) disruption of homophilic PCDH1 interactions, thereby weakening cell-cell contacts, and/or (ii) induction of signalling through PCDH1 and related δ1-protocadherins, or perhaps cadherin family members, thereby initiating a cellular response. In support of the first possibility, it is of interest to compare this finding to the function of soluble E-Cadherin fragments (sECad). sEcad was shown to participate in cell-migration (28) by homophilic binding to the E-cadherin/β-catenin complex, followed by the disruption of cell-cell adhesion

of E-cadherin molecules (29). With respect to the second possible function, several studies have shown that protocadherins can form heterophilic adhesion complexes by interacting in cis with cadherins (PCDH19 - N-cadherin (30), Arcadlin (rat PCDH8 ortholog) - N-cadherin (31), Pcdh1/Pcdh7/Pcdh9 (zebrafish) - N-cadherin (32)). Here, they either co-operate through heterophilic adhesion (30) or abrogate adhesion (31). As these interactions were identified with artificial fusion proteins in overexpression systems, future studies will need to determine whether soluble protocadherins also exist *in vivo* and can perform these functions. Altogether, based on these studies we suggest that sPCDH1 may positively or negatively influence PCDH1 function or the activity of other (proto)cadherins by homo or -heterophilic interactions with adhesion complexes in the extracellular compartment.

In the intracellular compartment we observed the presence of several small molecular weight PCDH1 proteins of 52, 50, 48 and 37kD, carrying the conserved signalling domains also found in full-length isoform 2. These intracellular PCDH1 protein products may also arise from translation of alternatively spliced mRNA transcripts, similar to the third isoform of *Pcdh1* we identified by RACE in mouse lung tissue (see Chapter 5), or from posttranslational mechanisms such as proteolytic cleavage of the intracellular tail of the full-length isoform 2 of PCDH1. This latter process has been described for γ-protocadherins (26), and Protocadherin-12 (PCDH12, (27)), where ADAM10 and γ-secretase-mediated cleavage resulted in the release of one soluble extracellular product and two similar sized intracellular products. One intracellular fragment was membrane-bound and was generated by ADAM10 processing of full-length γ-protocadherin or PCDH12. This fragment served as substrate for γ-secretases to generate a second cytoplasmic fragment (26, 27). The largest of the four intracellular PCDH1 fragments, the 52kD fragment, was present in both the cytoplasmic and the nuclear/membrane compartment. The presence of the 52kD fragment in the nuclear/membrane fraction was paralleled by an increase of the 52kD fragment in the cytoplasmic fraction, with highest expression at day 5 in a 16HBE confluency model. As we previously identified a putative isoform 3 of a similar size ((15), Chapter 5), we therefore hypothesize that the cytoplasmic 52kD fragment corresponds to this novel isoform and may translocate from cytoplasm to the nucleus. Alternatively, as tyrosine and serine phosphorylation sites were detected in the shared region of PCDH1 isoforms 1, 2 and 3 (33, 34), the 52kD fragment may arise due to phosphorylation of the processed 48kD fragment in the cytoplasm (see Figure 8). Furthermore, we detected slightly smaller 50kD, 48kD and 37kD fragments. For PCDH1 six lysine residues were identified in the constant shared region

of PCDH1 isoforms 1, 2 and 3 that may be targeted for ubiquitination, and subsequent breakdown by the proteasome (35). This proteasomal breakdown of full-length PCDH1 proteins may generate several smaller PCDH1 protein products. The 50kD, 48kD and 37kD fragments of PCDH1 are most abundant in the nuclear/membrane fraction. Our approach did not enable us to separate nuclear and membrane compartments. However, using immunofluorescence and confocal imaging, we confirmed the presence of PCDH1 isoforms 2 and/or 3 in the cytoplasm, and at the nuclear membrane, but not in the nucleus itself. Interestingly, PCDH1 isoform 1 localized to the apical compartment of 16HBE cells, and thus shows a distinct localization from isoforms 2/3. As isoform 1 also lacks CMs 1-3 and a PDZ-BS motif present in isoforms 2/3, these results suggest that these isoforms may mediate different signalling functions. Previously, it was shown for γ-protocadherins that the cytoplasmic fragment may translocate to the nucleus, but was mostly rapidly degraded by the proteasome (21). We speculate that similar processing may take place for PCDH1: the 170kD full-length PCDH1 isoform 2 may be subject to metalloprotease and γ-secretase processing, resulting in soluble 120kD sPCDH1 and membrane bound 50kD and 48kD fragments. The 48 kD fragment may reside at the nuclear membrane, where it is rapidly degraded by the nuclear proteasome (35, 36), resulting in 37kD and smaller breakdown products (see Figure 8 for a schematic overview). Further experiments are planned in our laboratory to investigate this possibility.

Figure 8: Model of interaction of PCDH1 isoforms with SMAD3
Schematic overview of the hypotheses regarding the processing of PCDH1 isoforms and their interaction with SMAD3. PCDH1 extracellular (sPCDH1) and intracellular protein products (37-52kD) may arise by shedding (depicted by scissors), alternative splicing (depicted by iso3 transcription in nucleus) and / or phosphorylation events (depicted by (P)) (left panel). Once generated, the sPCDH1 fragment resides in the extracellular compartment, while the intracellular 48-52kD fragments may either stay in the cytoplasm or translocate to the nucleus or nuclear membrane (middle panel). PCDH1 isoforms may bind to SMAD3 using the SMAD3 binding element (SBE) present in the constant shared region of all isoforms. This interaction may take place either in the cytoplasm or in the nucleus to affect SMAD3 activation/translocation or gene-transcription (right panel). CTF = C-terminal fragment.

PCDH1 processing:
1. Shedding
2. Alternative splicing
3. Phosphorylation

PCDH1 signaling:
1. Extracellular: sPCDH1 action
2. Intracellular: Cytoplasmic / nuclear action of CTFs

SMAD3 interaction:
1. Cytoplasm using SBE
2. Nucleus using SBE

Our time-range model of 16HBE is characterized by an increase in confluence that is associated with a strong rise in transepithelial resistance and the build-up of adherens and tight junctions, for which the junctional adhesion complex plays a critical role (23, 37). The formation of adhesion complexes is paralleled by the translocation of junctional adhesion molecules, like E-cadherin and ZO-1, from the cytosol to the membrane (38). Protocadherin-1 has been shown to mediate weak cell-cell adhesion (18), suggesting a possible role as adhesion molecule. We therefore compared PCDH1 isoforms 1 and 2 levels with E-cadherin levels in our fractions. To our surprise we did not observe a decrease in E-cadherin levels in both fractions, but rather an increase over time, suggesting that E-cadherin expression levels are upregulated with increasing 16HBE confluency (Figure 4A). Levels of PCDH1 isoform 2 increased in the cytoplasmic fraction but remained constant in the nuclear/membrane fraction, while isoform 1 levels did not show a clear trend for in- or decrease in both fractions. Remarkably, total full-length isoform levels (EC7-domain ab) decreased over time in nuclear/membrane and cytoplasmic fractions (Figure 4). We cannot fully explain the discrepancies between the different levels detected by these PCDH1 antibodies, although we acknowledge that part of the PCDH1 proteins may appear in the discarded insoluble fraction. Interestingly, we observed an apical localization of PCDH1 isoform 1 but not isoform 2 proteins by confocal microscopy. Furthermore, both isoforms 1 and 2 showed a staining pattern different from claudin-3, a tight junction molecule (Figure 5). The apical compartment is a separate compartment from adherens and tight-junction membrane-proximal compartments (39). We therefore conclude that PCDH1 isoforms localize differently than adherens and tight junction molecules like E-cadherin and Claudin-3. We hypothesize that PCDH1 isoform 1 localizes to the apical compartment of the cell over time, which after fractionation resides in the insoluble fraction, and that, together with a slight increase in cytoplasmic levels and constant nuclear levels of PCDH1 isoform 2, therefore total isoform levels decrease. Further experiments will be performed directed at immunohistochemical localization of PCDH1 isoforms during mucociliary differentiation.

We are the first to show a direct interaction of PCDH1 with SMAD3 at endogenous levels in a bronchial epithelial cell line. Since both genes are associated with asthma in genetic studies, we propose that the PCDH1/SMAD3 interaction constitutes a novel pathway in asthma development. We identified this interaction with both PCDH1 isoforms. Therefore the SMAD3 binding element is probably located in the shared, constant region of isoforms 1 and 2, just below the transmembrane-domain, and not in the conserved signalling domains

unique to isoforms 2 and 3. This shared element is also present in the novel isoform 3, and contains several serine and tyrosine residues that have been found to be phosphorylated (33, 34) consistent with participation in signal transduction (see Figure 8).

SMAD3 is an intracellular molecule that translates extracellular signals generated by TGFβ1 and activin-A (13) into specific gene-transcription by binding to SMAD4, followed by nuclear translocation (14). The gene transcriptional profile induced by TGFβ1 is associated with epithelial to mesenchymal transition (EMT) of several epithelial cell-types (40). During EMT adhesion molecules like E-cadherin are down-regulated, while α smooth muscle actin is upregulated to induce a migratory mesenchymal cell-type that produces extracellular matrix components (40). Interestingly, EMT was shown to be more severe in cultured asthmatic airway epithelial cells than in epithelial cells of healthy controls, and was shown to be dependent on SMAD3 (41). Previously we observed a clear association of PCDH1 mRNA and protein expression levels with mucociliary differentiation of bronchial epithelial cells. A differentiated cellular phenotype requires the establishment of adherens and tight-junctions, structures which are abrogated in EMT to induce cellular migration (40). We hypothesize that PCDH1 inhibits SMAD3 signalling, either in the cytoplasm, where it can inhibit activation or translocation of SMAD3, or in the nucleus where it may act as a transcriptional co-repressor (42). Thereby EMT is prevented and the expression of junctional proteins needed for a differentiated phenotype is maintained.

SMAD3 may additionally play a role in wound healing, as several studies showed that knock-down or knock-out of SMAD3 increased the re-epithelialization rate and subsequent wound healing of the skin of mice (43, 44). Interestingly, in a skin keratinocyte wounding model PCDH1 was upregulated during wound-healing (45). Furthermore, we previously showed that PCDH1 is upregulated during epithelial differentiation (15). Therefore, it is tempting to speculate that increased PCDH1 expression levels can inhibit SMAD3 signalling and thereby increase wound-healing or epithelial repair.

Altogether, by interacting with SMAD3, PCDH1 may play a role in EMT or epithelial repair. Dysfunction of the PCDH1 / SMAD3 pathway in the asthmatic airway epithelium may lead to increased EMT and exaggerated repair responses in asthma. Thereby these genes may co-operatively contribute to remodelling of the asthmatic airways.

We also need to consider some limitations of our study. We detected 50, 48, and 37kD fragments in the nuclear fraction, but this fraction also contains membrane proteins. The complementary fluorescence staining revealed a nuclear membrane localization that may

origin both from isoforms 2 and 3. Therefore, we are unable to identify the exact location of the 50, 48, and 37kD fragments. We are confident that these protein fragments orginate from PCDH1, but we acknowledge that their identity needs to be revealed by proteomic analysis. We did identify an endogenous interaction of both PCDH1 main isoforms with SMAD3. But as these interactions were detected in the 16HBE bronchial epithelial cell-line, we need to confirm these results in primary bronchial epithelial cells, and test whether this is different between those of asthmatics patients and healthy controls. We detected low SMAD3 protein levels after precipitation of Protocadherin-1 protein complexes, and *vice versa*. This might indicate that the interaction between PCDH1 and SMAD3 is either temporal or weak, or only involves part of the total protein pool available within the cells. Furthermore, the localization of the interaction is unknown. Therefore, we need to determine the relevance of this interaction using functional assays, like SMAD3 reporter assays, and during cellular transition processes like EMT, mucociliary differentiation, and epithelial repair. Furthermore, we suggest elucidating the localization of the interactions using co-localization studies.

In conclusion, we identified that besides cell-cell adhesion, PCDH1 may function as a signalling molecule. PCDH1 may participate in signalling by its 120kD sPCDH1 isoform, and via intracellular 48-52kD protein products that may participate in nuclear gene-transcription. Furthermore we identified an interaction of PCDH1 isoforms with SMAD3. Thereby we physically connect two asthma genes, and provide a new potential mechanism for asthma pathogenesis.

REFERENCES

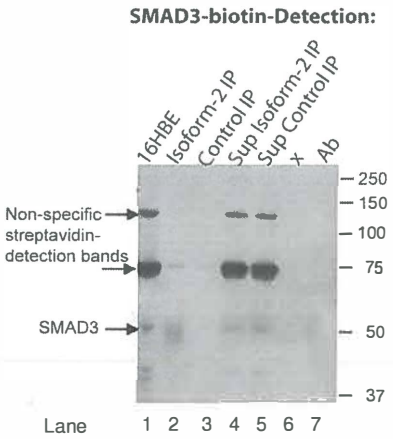
1. Braman SS. The global burden of asthma. Chest. 2006 Jul;130(1 Suppl):4S-12S.
2. Koppelman GH. Gene by environment interaction in asthma. Curr Allergy Asthma Rep. 2006;6(2):103-11.
3. Ober C, Yao TC. The genetics of asthma and allergic disease: A 21st century perspective. Immunol Rev. 2011;242(1):10-30.
4. Van Eerdewegh P, Little RD, Dupuis J, Del Mastro RG, Falls K, Simon J, et al. Association of the ADAM33 gene with asthma and bronchial hyperresponsiveness. Nature. 2002;418(6896):426-30.
5. Laitinen T, Polvi A, Rydman P, Vendelin J, Pulkkinen V, Salmikangas P, et al. Characterization of a common susceptibility locus for asthma-related traits. Science. 2004;304(5668):300-4.
6. Koppelman GH, Meyers DA, Howard TD, Zheng SL, Hawkins GA, Ampleford EJ, et al. Identification of PCDH1 as a novel susceptibility gene for bronchial hyperresponsiveness. Am J Respir Crit Care Med. 2009; 180(10):929-35.
7. Liew FY, Pitman NI, McInnes IB. Disease-associated functions of IL-33: The new kid in the IL-1 family. Nat Rev Immunol. 2010;10(2):103-10.
8. Orsmark-Pietras C, Melen E, Vendelin J, Bruce S, Laitinen A, Laitinen LA, et al. Biological and genetic interaction between tenascin C and neuropeptide S receptor 1 in allergic diseases. Hum Mol Genet. 2008; 17(11):1673-82.
9. Colland F, Jacq X, Trouplin V, Mougin C, Groizeleau C, Hamburger A, et al. Functional proteomics mapping of a human signaling pathway. Genome Res. 2004;14(7):1324-32.
10. Rual JF, Venkatesan K, Hao T, Hirozane-Kishikawa T, Dricot A, Li N, et al. Towards a proteome-scale map of the human protein-protein interaction network. Nature. 2005;437(7062):1173-8.

11. Luukko K, Ylikorkala A, Makela TP. Developmentally regulated expression of Smad3, Smad4, Smad6, and Smad7 involved in TGF-beta signaling. Mech Dev. 2001;101(1-2):209-12.
12. Elyaman W, Bassil R, Bradshaw EM, Orent W, Lahoud Y, Zhu B, et al. Notch receptors and Smad3 signaling cooperate in the induction of interleukin-9-producing T cells. Immunity. 2012;36(4):623-34.
13. Kariyawasam HH, Semitekolou M, Robinson DS, Xanthou G. Activin-A: A novel critical regulator of allergic asthma. Clin Exp Allergy. 2011;41(11):1505-14.
14. Brown KA, Pietenpol JA, Moses HL. A tale of two proteins: Differential roles and regulation of Smad2 and Smad3 in TGF-beta signaling. J Cell Biochem. 2007;101(1):9-33.
15. Koning H, Sayers I, Stewart CE, de Jong D, Ten Hacken NH, Postma DS, et al. Characterization of protocadherin-1 expression in primary bronchial epithelial cells: Association with epithelial cell differentiation. FASEB J. 2012;26(1):439-48.
16. Vanhalst K, Kools P, Staes K, van Roy F, Redies C. Delta-protocadherins: A gene family expressed differentially in the mouse brain. Cell Mol Life Sci. 2005;62(11):1247-59.
17. Hormi-Carver KK, Shi W, Liu CW, Berndt N. Protein phosphatase 1alpha is required for murine lung growth and morphogenesis. Dev Dyn. 2004;229(4):791-801.
18. Sano K, Tanihara H, Heimark RL, Obata S, Davidson M, St John T, et al. Protocadherins: A large family of cadherin-related molecules in central nervous system. EMBO J. 1993;12(6):2249-56.
19. Cavallaro U, Dejana E. Adhesion molecule signalling: Not always a sticky business. Nat Rev Mol Cell Biol. 2011;12(3):189-97.
20. Maretzky T, Reiss K, Ludwig A, Buchholz J, Scholz F, Proksch E, et al. ADAM10 mediates E-cadherin shedding and regulates epithelial cell-cell adhesion, migration, and beta-catenin translocation. Proc Natl Acad Sci U S A. 2005 28;102(26):9182-7.
21. Haas IG, Frank M, Veron N, Kemler R. Presenilin-dependent processing and nuclear function of gamma-protocadherins. J Biol Chem. 2005;280(10):9313-9.
22. Rouget-Quermalet V, Giustiniani J, Marie-Cardine A, Beaud G, Besnard F, Loyaux D, et al. Protocadherin 15 (PCDH15): A new secreted isoform and a potential marker for NK/T cell lymphomas. Oncogene. 2006;25(19):2807-11.
23. Heijink IH, Brandenburg SM, Noordhoek JA, Postma DS, Slebos DJ, van Oosterhout AJ. Characterisation of cell adhesion in airway epithelial cell types using electric cell-substrate impedance sensing. Eur Respir J. 2010;35(4):894-903.
24. Schreiber E, Matthias P, Muller MM, Schaffner W. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res. 1989;17(15):6419.
25. Nicolas FJ, De Bosscher K, Schmierer B, Hill CS. Analysis of smad nucleocytoplasmic shuttling in living cells. J Cell Sci. 2004;117(Pt 18):4113-25.
26. Reiss K, Maretzky T, Haas IG, Schulte M, Ludwig A, Frank M, et al. Regulated ADAM10-dependent ectodomain shedding of gamma-protocadherin C3 modulates cell-cell adhesion. J Biol Chem. 2006; 281(31):21735-44.
27. Bouillot S, Tillet E, Carmona G, Prandini MH, Gauchez AS, Hoffmann P, et al. Protocadherin-12 cleavage is a regulated process mediated by ADAM10 protein: Evidence of shedding up-regulation in pre-eclampsia. J Biol Chem. 2011;286(17):15195-204.
28. Ryniers F, Stove C, Goethals M, Brackenier L, Noe V, Bracke M, et al. Plasmin produces an E-cadherin fragment that stimulates cancer cell invasion. Biol Chem. 2002;383(1):159-65.
29. Noe V, Willems J, Vandekerckhove J, Roy FV, Bruyneel E, Mareel M. Inhibition of adhesion and induction of epithelial cell invasion by HAV-containing E-cadherin-specific peptides. J Cell Sci. 1999;112 (Pt 1)(Pt 1):127-35.
30. Emond MR, Biswas S, Blevins CJ, Jontes JD. A complex of protocadherin-19 and N-cadherin mediates a novel mechanism of cell adhesion. J Cell Biol. 2011;195(7):1115-21.
31. Yasuda S, Tanaka H, Sugiura H, Okamura K, Sakaguchi T, Tran U, et al. Activity-induced protocadherin arcadlin regulates dendritic spine number by triggering N-cadherin endocytosis via TAO2beta and p38 MAP kinases. Neuron. 2007;56(3):456-71.
32. Blevins CJ, Emond MR, Biswas S, Jontes JD. Differential expression, alternative splicing, and adhesive properties of the zebrafish delta1-protocadherins. Neuroscience. 2011;199:523-34.
33. Rush J, Moritz A, Lee KA, Guo A, Goss VL, Spek EJ, et al. Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. Nat Biotechnol. 2005;23(1):94-101.
34. Dephoure N, Zhou C, Villen J, Beausoleil SA, Bakalarski CE, Elledge SJ, et al. A quantitative atlas of mitotic phosphorylation. Proc Natl Acad Sci U S A. 2008;105(31):10762-7.
35. Kim W, Bennett EJ, Huttlin EL, Guo A, Li J, Possemato A, et al. Systematic and quantitative assessment of the ubiquitin-modified proteome. Mol Cell. 2011;44(2):325-40.
36. von Mikecz A. The nuclear ubiquitin-proteasome system. J Cell Sci. 2006;119(Pt 10):1977-84.

37. Cozens AL, Yezzi MJ, Kunzelmann K, Ohrui T, Chin L, Eng K, et al. CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. Am J Respir Cell Mol Biol. 1994;10(1):38-47.
38. Nawijn MC, Hackett TL, Postma DS, van Oosterhout AJ, Heijink IH. E-cadherin: Gatekeeper of airway mucosa and allergic sensitization. Trends Immunol. 2011;32(6):248-55.
39. Schuck S, Simons K. Polarized sorting in epithelial cells: Raft clustering and the biogenesis of the apical membrane. J Cell Sci. 2004;117(Pt 25):5955-64.
40. Hackett TL. Epithelial-mesenchymal transition in the pathophysiology of airway remodelling in asthma. Curr Opin Allergy Clin Immunol. 2012;12(1):53-9.
41. Hackett TL, Warner SM, Stefanowicz D, Shaheen F, Pechkovsky DV, Murray LA, et al. Induction of epithelial-mesenchymal transition in primary airway epithelial cells from patients with asthma by transforming growth factor-beta1. Am J Respir Crit Care Med. 2009 15;180(2):122-33.
42. Vandewalle C, Comijn J, De Craene B, Vermassen P, Bruyneel E, Andersen H, et al. SIP1/ZEB2 induces EMT by repressing genes of different epithelial cell-cell junctions. Nucleic Acids Res. 2005;33(20):6566-78.
43. Flanders KC, Major CD, Arabshahi A, Aburime EE, Okada MH, Fujii M, et al. Interference with transforming growth factor-beta/ Smad3 signaling results in accelerated healing of wounds in previously irradiated skin. Am J Pathol. 2003;163(6):2247-57.
44. Ashcroft GS, Yang X, Glick AB, Weinstein M, Letterio JL, Mizel DE, et al. Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. Nat Cell Biol. 1999;1(5):260-6.
45. Fitsialos G, Chassot AA, Turchi L, Dayem MA, LeBrigand K, Moreilhon C, et al. Transcriptional signature of epidermal keratinocytes subjected to in vitro scratch wounding reveals selective roles for ERK1/2, p38, and phosphatidylinositol 3-kinase signaling pathways. J Biol Chem. 2007 18;282(20):15090-102.

SUPPLEMENTARY DATA

Supplementary Figure E1: Specificity of Biotin-labelled SMAD3 antibody
The SMAD3-detection antibody was biotin-labelled to prevent detection of IgG-molecules at 50kD size. No non-specific IgG-band was detected in the lane where only antibody (lane 7, ab) was loaded, proving that no IgG-molecules are being detected using the SMAD3-detection antibody. This implies that SMAD3 is detected after immunoprecipitation of PCDH1 isoform 2 at 50kD (lane 2). IP=immunoprecipitation, x = empty lane, Ab = IgG antibody.



Chapter 7

Summary, Discussion and Future Perspectives

OVERVIEW

Major advances in asthma genetics have resulted in the identification of many novel asthma susceptibility loci. In order to further characterize the contribution of these genes to asthma pathogenesis, functional studies are required. This thesis describes the results of our investigations into the expression and regulation of *Protocadherin-1* (*PCDH1*), a novel gene associated with bronchial hyperresponsiveness and asthma. In this chapter we first summarize the data of *Chapters 2* to *6* of this thesis, followed by the interpretation of our findings. Finally, we provide suggestions for future research.

SUMMARY

In *Chapter 2* we describe the discovery of *PCDH1* as a novel gene for bronchial hyperresponsiveness (BHR) and asthma, and provide a basic characterization of its expression pattern. First linkage analysis was performed to identify the gene(s) responsible for the association of the chromosomal region 5q31-33 with asthma and BHR (1). Interestingly, a strong linkage signal was detected in families exposed to environmental tobacco smoke (ETS). Further fine-mapping of a 250kb region surrounding the marker that was most strongly associated with BHR resulted in the identification of *PCDH1*. The observed association of *PCDH1* with BHR was replicated in seven populations from the Netherlands, the United States and the United Kingdom, both in subjects with asthma and in the general population. We investigated the *PCDH1* mRNA expression pattern in a range of cell types, and identified high mRNA and protein expression in primary bronchial epithelial cells and the 16HBE bronchial epithelial cell line, while low mRNA expression levels were found in fibroblasts and peripheral blood mononuclear cells. The expression of Protocadherin-1 isoforms in bronchial epithelial cells was confirmed on protein level and in human lung tissue, and suggests a role for PCDH1 in the epithelial barrier.

Together our data identify *PCDH1* as a novel susceptibility gene for BHR, both in the asthmatic and general populations. As BHR is a hallmark of asthma, *PCDH1* may thereby influence asthma susceptibility via BHR. Thus, for the first time we implicate a member of the δ1-protocadherin family in BHR and asthma.

In *Chapter 3* we aim to identify whether *PCDH1* polymorphisms previously associated with asthma also associate with eczema. Asthma and eczema have a common

genetic background, but also disease specific genetic factors exist (2). *PCDH1* is located on chromosome 5q31, a region linked to both asthma and eczema (1). We report an association of one *PCDH1* polymorhism Ala514Thr (rs3822357) with eczema in one birth cohort, whereas a second polymorphism, the insertion deletion polymorphism IVS3-116 associates with eczema in two independent birth cohorts.

Eczema is a disease of the skin, characterized by local inflammation, itching, redness, and increased trans-epidermal water loss (3). To interpret a shared genetic susceptibility of asthma and eczema, it is of interest to compare the epithelial barrier of the airways and the skin. The skin barrier is built up by a skin-specific epithelial cell type, the keratinocyte, while the airway epithelial barrier is built up by bronchial epithelial cells. The skin epithelium forms two separate barriers. The first barrier is the stratum corneum, consisting of multiple layers of keratinocytes without nuclei (corneocytes), and protects from dehydration and injury (4). Below the stratum corneum several layers of intact keratinocytes form the second physical barrier by close attachment via tight junctions (5), and they control the passage of water, ions and solutes, and microbes and viruses. In contrast to the skin, the airway epithelium has only one barrier: stratified ciliated epithelial cells that are closely attached via adherens and tight junctions and connect with the basal epithelial cell layer. Stratified epithelial cells form an ion selective barrier, and a size selective barrier against inhaled environmental agents (6). A defect in these skin and airway wall barriers may be an underlying cause of eczema and asthma. Indeed, several eczema susceptibility genes have been identified that are expressed in the stratum corneum, the first barrier, including Filaggrin (*FLG*), (7); and small proline rich protein 3 (*SPRR3*, (8), or in the keratinocytes, the second barrier, including *Claudin-1*, (5). *FLG* and *SPRR3* are located in the epidermal differentiation complex (EDC) locus on chromosome 1q21.3. The EDC family of genes is involved in correct maturation and cornification of the skin barrier (8). Claudin-1 is a structural tight junction protein, and its down regulation may induce loss of tight junction function and facilitate increased entry of microbes or viruses (5). Furthermore, a recent GWAS meta-analysis has identified *OVOL1* as an eczema gene that is involved in the regulation and differentiation of the male germinal epithelium and epidermal tissues (9). Thus several susceptibility genes for eczema are identified that may play a role in the formation and integrity of the skin barrier.

In *Chapter 3* we show that *PCDH1* polymorphisms associate both with eczema and asthma. Moreover, we report that *PCDH1* mRNA and protein isoforms are expressed in keratinocytes and bronchial epithelial cells. Thus, we propose that PCDH1 has a function in a pathway that contributes to both asthma and eczema. Interestingly, *IL1RL1,* a gene encoding

the IL-33 - receptor, is also associated with both eczema (10) and asthma (11). As IL-33 is an alarmin that is produced by both keratinocytes and bronchial epithelial cells (12), the IL1RL1-IL33 pathway constitutes a novel disease associated pathway at the interface of structural (keratinocytes, bronchial epithelial cells) and immune cells.

We speculate that variation in shared genes like *PCDH1* and *IL1RL1* may contribute to the observed barrier defects (*PCDH1*), and changes in IL1RL1-IL-33 signalling (*IL1RL1*) in both diseases, while variation in diseases specific genes contribute to eczema and asthma separately. Further genetic studies will have to replicate our findings on *PCDH1* and to identify the function of PCDH1 in both skin and airway epithelium.

In *Chapter 4* we perform an in-dept analysis of *PCDH1* expression levels in cultured and *ex vivo* primary bronchial epithelial cells of asthma patients, and in differentiated bronchial epithelial cells of healthy subjects. We identify complex alternative splicing patterns of *PCDH1*. First, we report novel 5'exons that differ between the two main isoforms of *PCDH1*, suggesting alternative promoter usage. In addition, a CpG-Island is detected that surrounds exon 1A. Second, we show the identification of a transcript that lacks five extracellular cadherin repeats (ECs), and only contains two ECs, the transmembrane domain and the intracellular domains, and named it isoform 3. The intracellular tail of PCDH1 isoform 2 is characterized by three conserved motifs (CM1-3) and a C-terminal PDZ-domain binding site (PDZ-BS). Third, we report of novel transcripts that lack the CM2 domain and PDZ-BS and thus may have other intracellular signalling properties than isoforms that express all conserved intracellular domains. These studies show that PCDH1 is subject to complex splicing patterns.

Importantly, we identify a clear association of *PCDH1* mRNA and protein expression levels with epithelial differentiation of primary bronchial epithelial cells. This association of *PCDH1* expression levels with epithelial differentiation may not only be specific to bronchial epithelial cells, but also to primary cells in the skin, the keratinocytes. This is supported by observations on *PCDH1* mRNA expression in two keratinocyte datasets. First, *PCDH1* mRNA expression was 5.5-fold upregulated during confluence induced differentiation of normal human keratinocytes (13). Second, *PCDH1* mRNA was higher expressed in the more differentiated suprabasal keratinocytes, compared to progenitor-like basal keratinocytes (14). We conclude that PCDH1 is regulated during epithelial differentiation, but its exact function in differentiated epithelia needs further investigation.

In *Chapter 5* we aim to identify the *in vivo* regulation of *Pcdh1* in mouse lung by environmental exposures that increase BHR in experimental mouse models, and that are known risk factors for asthma development, such as housedust mite (HDM) (15) and cigarette smoke (CS) exposure (16). We investigate the regulation of Pcdh1 by HDM and CS in HDM-induced asthma models and acute and sub chronic CS-exposure models. To this end, we first assessed homology on the gene and mRNA transcripts between human *PCDH1* and mouse *Pcdh1*. We identified a remarkably high homology between mouse Pcdh1 and human PCDH1, both on RNA and protein level, and confirmed the existence in mice of a third isoform encoding intracellular domains (mouse isoform 3). The high similarity of PCDH1 in mice and men may indicate that PCDH1 performs an important function that has been conserved during evolution (17). In our chronic HDM exposure model we detected slightly lower PCDH1 protein expression levels after HDM exposure. Especially in airway epithelial cell layers that had undergone goblet cell metaplasia upon HDM exposure almost no PCDH1 staining was observed (*Chapter 5*). In addition, we observed an upregulation of *PCDH1* mRNA expression levels in the recovery phase after housedust mite exposure. These results may suggest that HDM down regulates PCDH1 expression levels and that PCDH1 expression is induced in the repair phase. In our CS-exposure mouse model, we observed that CS-exposure resulted in a direct and clear down regulation of PCDH1 expression levels in mouse lung. CS-exposure is known to affect the epithelial barrier by decreasing cell-cell contacts (18). We propose that subjects exposed to environmental tobacco smoke may, in the presence of susceptible *PCDH1* alleles, develop BHR and asthma, due to downregulation of PCDH1 and epithelial adhesion molecules and a subsequent impaired epithelial barrier function.

In *Chapter 6* we investigate whether PCDH1 could act as a signalling molecule by identifying the subcellular localization of its protein isoforms and its interaction with SMAD3. Since PCDH1 does not appear to be a classical adhesion molecule as its adhesion properties are weak (19) or not detectable (20), it is important to consider alternate functions, such as signalling functions. PCDH1 isoforms 1, 2, and 3 contain conserved tyrosine and serine residues in their intracellular tail that may be phosphorylated (21, 22). Furthermore, conserved motifs (CM1-3) and a PDZ-binding site are present in the intracellular tail of isoform 2, motifs that are shared with isoform 3. Importantly, two yeast-2-hybrid studies using protein overexpressions suggest an interaction between PCDH1 and SMAD3 (23, 24). Therefore, we were interested whether PCDH1 may play a role in downstream signalling, by

assessing PCDH1 isoform expression in the extracellular and intracellular compartments, and its interaction with SMAD3. We detected a novel extracellular soluble PCDH1 protein product (sPCDH1), and multiple intracellular protein products (*Chapter 6*), that may play a role in signalling. Further proteomic analysis is needed in order to identify the origin of these protein products.

Interestingly, we observed an interaction of both PCDH1 isoforms 1 and 2 with SMAD3, suggesting that PCDH1 can act as a signalling molecule. *SMAD3* has been identified as an asthma gene by the genome-wide meta-analysis from the GABRIEL consortium (11). SMAD3 is a key downstream molecule in the TGFβ-pathway, and is a critical factor for epithelial to mesenchymal transitions (EMT) and epithelial repair responses (25). The asthmatic epithelium is more sensitive to TGFβ-induced EMT than epithelium of healthy controls (*in vitro*, (25)), but no evidence for EMT in airways of asthmatic patients has yet been identified (26). However, a recent *in vivo* study showed that prolonged exposure of the mouse airway to HDM induces an increase in epithelial derived mesenchymal cells in mouse airways, a finding paralleled by an increase in TGFβ in the broncheoalveolar lavage fluid. This may suggest that airway epithelial cells of mice exposed to HDM undergo EMT and indicate a possible role for TGFβ in this process (27). We propose that PCDH1 may inhibit TGFβ-induced EMT by interacting with SMAD3. Interestingly, several *in vivo* studies showed that knock-out of SMAD3 in mice resulted in an increased skin wound repair (28, 29). As we observed a physical interaction between PCDH1 and SMAD3, we propose that the sensitivity towards TGFβ-signalling is decreased in the normal situation due to high PCDH1 expression levels in differentiated epithelial cells. It is tempting to speculate that PCDH1 levels are dysregulated in a disease situation (i.e. asthma or eczema), followed by an increased sensititity towards TGFβ-signalling, and a chronic epithelial repair phenotype.

In conclusion, the physical interaction between the proteins encoded by two asthma susceptibility genes implicates the existence of a novel pathway in asthma pathogenesis in which these two proteins play a central role.

DISCUSSION

When we integrate the findings of *Chapters 2* to *6*, we can discern four main topics for further study; the relevance of δ1-protocadherins in asthma, epigenetic regulation of

protocadherins, PCDH1 signalling and potential functions of PCDH1 related to asthma development.

The role of δ1-protocadherins in asthma

We identified *PCDH1* as a novel susceptibility gene for BHR and asthma. PCDH1 belongs to the δ1-protocadherin sub-family, consisting of PCDH1, PCDH7, PCDH9 and PCDH11, within the cadherin superfamily of adhesion molecules. The δ1-protocadherin family is part of the non-clustered protocadherin family, characterized by the presence of seven extracellular cadherin repeats (30). δ1-protocadherin genes display a high level of alternative splicing, resulting in isoforms that encode alternative cytoplasmic domains (31). δ1-protocadherin family members are expressed in a range of tissues, such as brain, heart, liver, kidney and lung (32, 33). Interestingly, a recent GWAS meta-analysis suggested two other δ1-protocadherins, *PCDH7* and *PCDH9*, to be associated with lung function in asthma patients (34). Furthermore, PCDH1, PCDH7 and PCDH9 are upregulated during epithelial differentiation in an air liquid interface culture model (35, 36). These studies suggest that multiple members of the δ1-protocadherin family expressed in airway epithelial cells may play a role in asthma susceptibility or severity as they are associated with two hallmarks of asthma: BHR and lung function. This supports further research into the exact role of the δ1-protocadherins in airway epithelial cell differentiation and asthma.

Epigenetic regulation of Protocadherin-1

We identified a strong linkage signal of *PCDH1* with BHR in families exposed to environmental tobacco smoke compared to unexposed families (37). In addition, we observed a decrease of *Pcdh1* expression levels after cigarette smoke exposure in mice, as early as 6 hours after exposure. Of note, *PCDH1* gene exon 1A is situated in a CpG-Island that may be methylated and that is conserved between man and mouse. Therefore the mechanism behind the decrease in *Pcdh1* expression levels may encompass epigenetic changes induced by cigarette smoke, or constitute a direct transcriptional effect. Interestingly, differential methylation of another protocadherin, *PCDH20*, was observed in DNA isolated from sputum cells of asthma subjects who smoke compared to healthy smokers (38). These results suggest that methylation of *δ-Protocadherin* gene promoters may be a mechanism to regulate their expression levels. The regulatory effects of methylation, and the effects of cigarette smoke and other environmental stimuli that are known risk factors for asthma will be subject of further investigations.

Protocadherin-1 signalling

PCDH1 may perform intracellular signalling at different cellular compartments. Initially two main isoforms of PCDH1 were identified in the airway epithelium; a short 3 exon isoform and a long 5 exon isoform that additionally encodes an intracellular domain with conserved motifs (CM1-3, PDZ-BS). We investigated whether the two main transcripts and potential novel transcripts were expressed in bronchial epithelial cells. We confirmed the expression of the two main isoforms in bronchial epithelial cells and identified mRNA transcripts that lack the conserved motif CM2 and / or PDZ-BS, and thereby may influence signal transduction or its localization, as PDZ-BS can provide anchorage for PDZ-domain molecules for apical sorting (39). Furthermore we detected an isoform that lacks 5 (human isoform 3) or all (mouse isoform 3) extracellular cadherin repeats. These isoforms encode signalling domains and may directly be involved in downstream signalling.

Previously, research has shown that adhesion molecules like E-cadherin, and γ-protocadherins can be shed by metalloproteinases like A Disintegrin And Metalloproteinase 10 (ADAM10) and further proteolytically processed by γ-secretases (40, 41). Shedding of E-cadherin resulted in the generation of extracellular soluble E-cadherin (sEcad), while γ-secretase processing yielded intracellular C-terminal fragments. Extracellular sEcad was shown to stimulate migration of epithelial cells by inhibiting E-cadherin mediated adhesion complexes (40), while the intracellular E-cadherin fragment was shown to bind to β-catenin thereby inhibiting its signalling (42). γ-Protocadherins were also shed by ADAM10 (41). The resulting intracellular γ-protocadherin protein products were shown to reside in the nucleus to induce gene transcription (41). Therefore, besides by generating alternative transcripts with different signalling domains, we hypothesize that *PCDH1* may also be subject to post-translational processing by ADAMs and γ-secretases, possibly resulting in intracellular protein products that may participate in signalling. Indeed, we identified a soluble (s)PCDH1 in the culture supernatant of 16HBE bronchial epithelial cells, and a series of intracellular protein products in cytoplasmic and nuclear/membrane fractions. But as we previously discovered transcripts that lack extracellular domains (human and mouse isoform 3) the intracellular protein products may as well arise from alternative splicing. Alternatively, as conserved lysine residues were identified in the shared constant region of PCDH1 isoforms 1, 2 and 3 that may be ubiquitinated (43), these fragments can as well be generated by proteasomal breakdown. The function of the extracellular and intracellular protein products for PCDH1 is unknown. The presence of these fragments in different cellular compartments may implicate that they participate in signalling, but as it is unsure whether these fragments

are generated by ADAM/MMP-processing, alternative transcription or proteasomal breakdown, the exact mechanism remains to be elucidated.

In *Chapter 6* we identified that PCDH1 interacts with SMAD3. This is the second confirmed PCDH1 interacting protein, besides Protein Phosphatase 1α (PP1α) (31). The interaction of PCDH1 with PP1α is isoform specific, since PP1α interacts with the conserved CM3-motif, only present in PCDH1 isoforms 2 and 3. In contrast, the PCDH1-SMAD3 interaction is detected for both isoform 1 and 2, and therefore is thought to occur via the constant shared domain of isoforms 1 and 2 containing conserved serine and tyrosine residues that can be phosphorylated (21, 22).

Altogether, PCDH1 may perform signalling functions at three different levels; 1) by alternative splice-variants that express different signalling domains or lack extracellular cadherin-repeats, 2) by intracellular protein products potentially generated by post-translational processing, and 3) by interacting with downstream signalling partners like PP1α and SMAD3.

Potential implications of PCDH1 functions: role in adhesion, differentiation, EMT, and/or repair

In our first characterization of PCDH1 expression in the airway epithelium, we observed expression specifically at the apical sites of ciliated bronchial epithelial cells, but not in basal undifferentiated cells (37). Based on its expression pattern in the airway epithelium and observations that PCDH1 may perform (weak) adhesion functions (19), we hypothesized that PCDH1 may influence the epithelial barrier function. Protocadherin-1 contains several apical localization signals. First, a PDZ-domain binding site was detected (44), which may provide anchorage for transport to the apical domain. Second, five N-glycosylation sites have been predicted (http://www.cbs.dtu.dk/services/NetNGlyc/) which can function as apical localization signals (45). Interestingly, several other protocadherins also display apical localization, like γ-protocadherins (46), protocadherin-LKC/PCDH24 (47), and mu-protocadherin/CDHR5 (48). The function of the apical localization is currently unknown, but may include a role in the apical adhesion complex and / or the establishment of cell polarity.

The asthmatic epithelium is characterized by a decreased epithelial barrier function, and a lowered expression of junctional molecules, like ZO-1 and E-cadherin. The aforementioned protocadherins did not show co-localization with junctional molecules such as ZO-1. Our immunofluorescent localization studies on PCDH1 in a bronchial epithelial cell-line showed a punctuate supra-nuclear apical staining pattern for PCDH1 isoform 1, while

isoform 2 displayed a cytoplasmic filamentous staining pattern that concentrated around the nucleus. Furthermore their localization was different from Claudin-3, a tight-junction marker. We acknowledge that we need to determine the specificity of these PCDH1 stainings by PCDH1 knockdown and overexpression studies. Nevertheless, these results suggest that the role of PCDH1 is not consistent with a function in adhesion complexes. What other functions could PCDH1 perform besides cell-cell adhesion functions? We have described a correlation of PCDH1 expression levels with differentiation of bronchial epithelial cells (36), and as previously mentioned, similar results were obtained for PCDH7 and 9 in ALI cultures using micro-arrays (35), suggesting that δ1-protocadherins are highest expressed in ciliated bronchial epithelial cells. Indeed, from immunohistochemistry in mouse lung tissue we specifically identified expression of Pcdh1 in terminally differentiated ciliated epithelial cells, but no staining in goblet cells. Furthermore studies from model-organisms like Xenopus, Drosophila and mouse embryos regarding the function of protocadherin-othologs showed that protocadherins can coordinate tissue morphogenesis and cell-polarity (49, 50), but their function in human cells remains to be determined. Therefore, from these observations we can speculate that PCDH1 is not involved in cell-cell adhesion by a role in the apical adhesion complex, but rather acts in epithelial polarization or differentiation. Additional support for this stems from our investigations of PCDH1 as a candidate signalling molecule, as we observed an interaction of PCDH1 with SMAD3. SMAD3 is implicated in several processes like epithelial to mesenchymal transition (EMT) and wound repair (51). These processes are closely related as they are paralleled by transient loss of terminal differentiation of the epithelial cell and the (partial) adaptation of a more mesenchymal phenotype (EMT) (26), followed by migration of cells to the injured area and restoration of cell-cell contacts and barrier function. Finally a re-adaptation state is initiated that results in terminal differentiation of the cells (wound repair) (52).

PCDH1 may play a role in several stages of this process. First, PCDH1 may influence EMT by interacting with SMAD3. During TGFβ-induced EMT, the expression of adhesion-molecules like E-cadherin is down-regulated, while mesenchymal markers like α-smooth muscle actin, vimentin and fibronectin are upregulated. The activation of gene-transcription during the EMT process is dependent on the transcription factor SMAD3, and results in a migratory mesenchymal phenotype (25). We propose that PCDH1 levels are decreased in an asthmatic individual with susceptible *PCDH1* alleles, a process followed by an increase in free SMAD3 and subsequent increase in TGFβ-signalling, resulting in a chronic epithelial repair phenotype. Interestingly, besides TGFβ the related family member Activin-A may as

well signal via SMAD3. Activin-A is upregulated directly after epithelial injury, and is considered a potent inducer of epithelial repair (53). The exact role of Activin-A in the airway epithelium is just beginning to be discovered, but we speculate that by interacting with SMAD3, PCDH1 may as well influence Activin-A induced epithelial repair.

Second, PCDH1 may play a role in the final stage of epithelial repair: terminal differentiation of migrated epithelial cells. After migration of epithelial cells to a wounded area, residing epithelial cells need to obtain a terminal differentiated phenotype, such as a ciliated cell, in order to form a functional protective barrier. During epithelial differentiation there is an increase in PCDH1 expression levels. The increased PCDH1 expression levels may result in a decrease of SMAD3 protein levels that may participate in TGFβ-signalling, and therefore opposite transition processes (EMT) are halted. It is tempting to speculate that in the airway epithelium of asthma subjects with susceptible *PCDH1* alleles PCDH1 levels are dysregulated. As a consequence, an undifferentiated phenotype is maintained, characterized by a decreased build up of adherens and tight junctions. Together with a dysregulated TGFβ- or Activin-A signalling, this may result in a chronic epithelial repair phenotype and a weakened epithelial barrier. Subsequently, a weakened epithelial barrier may lead to an increased entrance of environmental factors like allergens and pathogens, thereby continuously activating the immune system leading to a chronically inflamed airway (54), and increased smooth muscle mass due to hypertrophy and/or hyperplasia of the smooth muscle layer that may contribute to BHR and asthma.

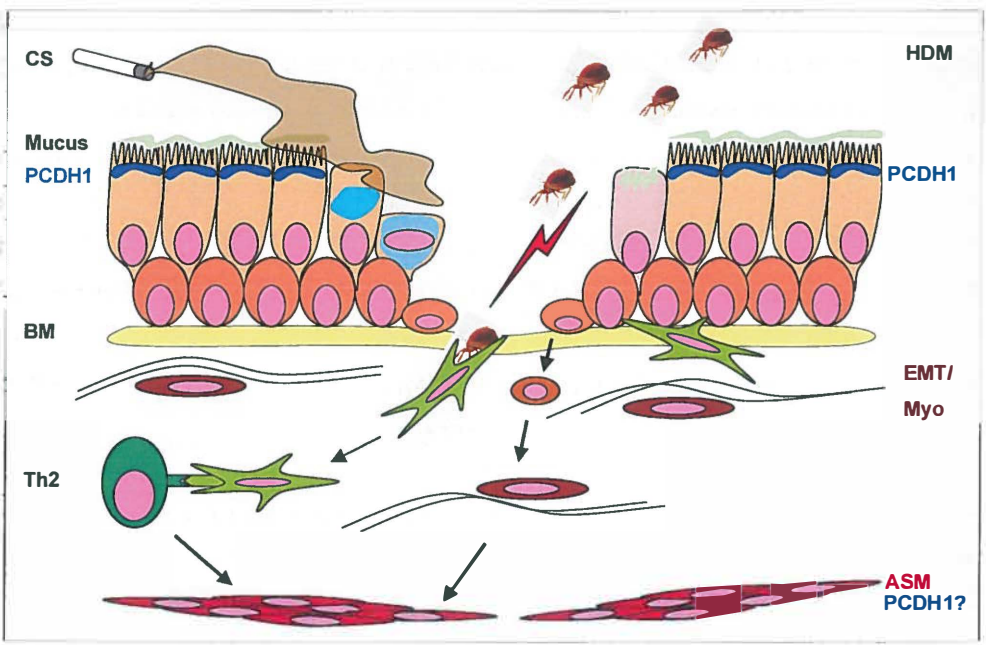
In conclusion, *PCDH1* is genetically associated with BHR, asthma and eczema. PCDH1 is highly expressed in differentiated bronchial epithelial cells, while its expression levels are lower in goblet cells or undifferentiated epithelial cells. Combined with observations using immunofluorescence localization studies, we show that PCDH1 is not associated with cellular adhesion complexes and may rather be involved in polarization or differentiation of airway epithelial cells. In addition PCDH1 may act as a signalling molecule as several intracellular protein products were identified that may be generated by ADAM/MMP processing or alternative splicing, and as it interacts with SMAD3. By influencing TGFβ-induced SMAD3 signalling PCDH1 may play an important role in EMT, wound repair and differentiation.

How can we integrate our current knowledge on PCDH1 function into our understanding of asthma? The epithelial barrier is compromised in asthma (55), which may in part be due to PCDH1 dysregulation, and may lead to an increased access of inhaled allergens

to the immune system promoting allergic sensitisation. Furthermore due to the weakened airway epithelium barrier function, the airway epithelium of asthma patients responds differently to environmental exposures like cigarette smoke, housedust mite, and viruses compared to healthy subjects by producing increased amounts of pro-inflammatory cytokines (6). Together with an increased availability of inhaled allergens this may lead to priming of the immune system towards a Th2-dominated allergic response and progression to a chronically inflamed phenotype. Environmental cigarette smoke or housedust mite exposure down regulate PCDH1 expression levels, potentially via epigenetic mechanisms, whereas the effect of viral exposures on PCDH1 expression remains unknown. Thereby these environmental factors may further compromise the epithelial barrier function, rendering subjects more susceptible for injuries. This may activate the EMTU, which may lead towards a continuous repair phenotype (52). Changes of the airway epithelium from a ciliated towards a goblet cell phenotype may lead to excessive mucus production, and airway narrowing (54). Components of structural cells of the airway (ECM deposition by (myo)fibroblasts and epithelial cells) and of immune cells (cytokines, growth factors) in the airway may induce hypertrophy and / or hyperplasia of the smooth muscle layer, leading to increased smooth muscle mass (56). As we identified expression of Pcdh1 in mice airway smooth muscle cells, the role of Pcdh1 in the increase in smooth muscle cell mass needs further investigation. Subjects with a specific genetic susceptibility in genes encoding epithelial factors (for example *PCDH1*) or immune functions may thus in combination with an increased exposure to harmful agents or physical stress (i.e. environment), eventually develop BHR and asthma (see Figure 1). If this is a basic mechanism underlying the initiation of asthma, future therapeutic interventions may be targeted at restoring the ciliated epithelial phenotype, to create a more stable epithelium that maintains a proper barrier function protective towards foreign agents, allergens included. Specifically, provided that PCDH1-SMAD3 signalling is impaired in the asthmatic epithelium, we speculate that stabilizing the PCDH1-SMAD3 interaction may decrease EMT or increase the repair potential of epithelial wounds and thus create a protected, differentiated epithelial phenotype. Future research is needed into the function of PCDH1, SMAD3, and their interaction in asthma pathogenesis.

Figure 1: Model: Cigarette smoke and House-dust mite exposure changes epithelial barrier by modulation of Protocadherin-1 expression levels

The epithelial barrier of the large airways consists of mainly basal and ciliated bronchial epithelial cells, located on top of the basement membrane (BM). Epithelial cells express PCDH1, which localizes to the apical site just below the brush border (apically localized, marked in blue). Cigarette smoke (CS) exposure may down-regulate PCDH1 and junctional adhesion molecules, thereby de-differentiating epithelial cells. House-dust mite (HDM) exposure may injure the epithelial layer or induce the formation of goblet cells (pink color), with no or low PCDH1 expression, and results in excessive mucus in the airway. Due to a dysregulated interaction of PCDH1 with SMAD3 in asthmatic subjects with susceptible PCDH1-alleles, epithelial repair remains unfinished and epithelial cells may in addition go into epithelial mesenchymal transition (EMT). The resulting migrating myofibroblasts (Myo) produce extracellular matrix components (ECM). Altogether, this may lead to airway epithelium with a continuous repair phenotype, and a subsequent weakened epithelial barrier. The weakened epithelial barrier may result in increased sensitization of the immune system. Together with increased cytokine and growth factor production by asthmatic epithelial cells this may lead to a chronic inflammatory response that is partly Th2-mediated. Components of structural cells of the airway (ECM) and immune cells (cytokines, growth factors) may induce hypertrophy and / or hyperplasia of the smooth muscle layer. Together with an excessive production of mucus in the airway, this may lead to BHR and asthma.



FUTURE PERSPECTIVES

Recent genome wide association studies identified 16 asthma loci with genome wide significance (57). This thesis provides a first characterization of a novel gene for BHR and

asthma with a yet unknown function, Protocadherin-1. The technical advances in the development of whole genome sequencing and parallel total RNA sequencing of lung tissue and its separate cell types like epithelial cells and smooth muscle cells will reveal novel disease associated genes and their corresponding transcriptome in the near future. Research into epigenetics will additionally identify another level of gene-regulation, which then needs to be combined into an integrative genomics approach towards asthma (58).

An increasing number of asthma susceptibility genes are being identified. There is now a need for the scientific community to prioritize functional genetic investigations of these susceptibility genes. These genes can be different per population, as US and Japanese GWAS-studies have identified population specific loci, like *PYHIN1* for afro-americans (59), and *USP38-GAB1* locus for the Japanese population (60). Moroever, a specific gene may be important in a certain subphenotype of asthma, e.g. BHR for *PCDH1*, or childhood onset asthma for *ORMDL3 / GSDMB* (61). In general we propose the following procedures after identification of a novel asthma gene (see Table I):

Table I: Strategy for functional analysis of a novel disease associated gene

Step	Action	Required tools / experiments
1.	Obtain background information	Literature and expression databases
2.	Generate and optimize toolbox	(q RT)-PCR assays, Overexpression plasmids, siRNA, antibodies, ELISAs
3.	Analyze expression in range of cell-types	Model cell-lines, primary cells and tissues
4.	Identify transcripts and protein isoforms, and investigate differences between healthy and disease, that may be regulated by the susceptibility SNPs	- Model cell-lines, primary cells and tissues - Identify eQTLs (62) in relevant cells and/or tissues
5.	Modulate gene expression to investigate gene function	*In vitro:* siRNAs, overexpressions, modulation of epigenetic signatures *In vivo:* KO and gene overexpressing mice
6.	Determine effect of environmental exposures on gene expression / function	Genetic epidemiological studies CS, HDM, particulate matter exposures in cell-lines, mouse-models
7.	Investigate how gene-variants modulate gene function	SNP specific functional assays
8.	Perform protein interaction and pathway analysis	Immunoprecipitation, micro-arrays, proteomics
9.	Investigate effect of modified gene-function with environmental exposures on previously identified (step 8) pathways	-qPCR/antibodies for siRNA / overexpressions: cell-systems -KO and overexpression mouse models -micro-array exposed vs non-exposed
10.	Identify and intervene upon dysfunctional pathways	Therapeutic intervention

eQTL = expression Quantitative Trait Locus (62); *KO = knock-out ; CS = cigarette smoke ; HDM = House-dust mite*

When we apply these steps to the research in the function of PCDH1, we can suggest several next steps:

- We determined PCDH1 expression levels in asthma subjects, but were not able to correlate PCDH1 genotypes to gene-expression (identify eQTLs) due to a low number of available cells/tissue from asthma subjects. An important first step is to investigate how and if PCDH1 non-coding (promoter) gene-variants induce changes in PCDH1 mRNA or protein expression levels, and perhaps gene methylation, by screening a larger population of epithelial cells from healthy and asthmatic subjects.
- We suggest to investigate the function of PCDH1 by siRNA experiments (*in vitro*) and KO-studies (*in vivo*). Outcomes of main interest are adhesion, epithelial polarization, differentiation and wound repair.
- KO and Pcdh1 isoform specific overexpression mouse models should be employed in order to investigate whether there is a changed sensitivity towards allergic sensitisation, asthma and BHR development, both with and without environmental exposures like HDM and CS.
- We observed a down-regulation of *Pcdh1* expression levels by CS *in vivo*. Therefore, further investigations into potential epigenetic regulation are needed.
- The significance of the PCDH1 - SMAD3 interaction should be further investigated. Specifically it should be investigated whether PCDH1 inhibits or stimulates TGFβ induced SMAD3-signalling. Furthermore it should be investigated whether PCDH1 is involved in Epithelial to Mesenchymal Transition (EMT) or wound repair.
- It is unknown whether PCDH1 is involved in other signalling pathways. Therefore co-immunoprecipitation experiments should be performed to confirm interactions with proposed interactors like PP1α and to identify potential novel interaction partners.
- As more protocadherin family members are associated with asthma or lung function we propose that the complete family of δ-protocadherins should be characterized in asthma and BHR.

Future investigations into the role of δ1-protocadherins, specifically PCDH1 may reveal novel pathways and interactions that are at the basis on asthma initiation.

REFERENCES

1. Postma DS, Bleecker ER, Amelung PJ, Holroyd KJ, Xu J, Panhuysen CI, et al. Genetic susceptibility to asthma--bronchial hyperresponsiveness coinherited with a major gene for atopy. N Engl J Med. 1995; 333(14):894-900.
2. van Beijsterveldt CE, Boomsma DI. Genetics of parentally reported asthma, eczema and rhinitis in 5-yr-old twins. Eur Respir J. 2007;29(3):516-21.
3. Biagini Myers JM, Khurana Hershey GK. Eczema in early life: Genetics, the skin barrier, and lessons learned from birth cohort studies. J Pediatr. 2010;157(5):704-14.
4. Jungersted JM, Hellgren LI, Jemec GB, Agner T. Lipids and skin barrier function--a clinical perspective. Contact Dermatitis. 2008;58(5):255-62.
5. De Benedetto A, Slifka MK, Rafaels NM, Kuo IH, Georas SN, Boguniewicz M, et al. Reductions in claudin-1 may enhance susceptibility to herpes simplex virus 1 infections in atopic dermatitis. J Allergy Clin Immunol. 2011;128(1):242,246.e5.
6. Nawijn MC, Hackett TL, Postma DS, van Oosterhout AJ, Heijink IH. E-cadherin: Gatekeeper of airway mucosa and allergic sensitization. Trends Immunol. 2011;32(6):248-55.
7. Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. Nat Genet. 2006;38(4):441-6.
8. Marenholz I, Rivera VA, Esparza-Gordillo J, Bauerfeind A, Lee-Kirsch MA, Ciechanowicz A, et al. Association screening in the epidermal differentiation complex (EDC) identifies an SPRR3 repeat number variant as a risk factor for eczema. J Invest Dermatol. 2011;131(8):1644-9.
9. Paternoster L, Standl M, Chen CM, Ramasamy A, Bonnelykke K, Duijts L, et al. Meta-analysis of genome-wide association studies identifies three new risk loci for atopic dermatitis. Nat Genet. 2011;44(2):187-92.
10. Shimizu M, Matsuda A, Yanagisawa K, Hirota T, Akahoshi M, Inomata N, et al. Functional SNPs in the distal promoter of the ST2 gene are associated with atopic dermatitis. Hum Mol Genet. 2005;14(19):2919-27.
11. Moffatt MF, Gut IG, Demenais F, Strachan DP, Bouzigon E, Heath S, et al. A large-scale, consortium-based genomewide association study of asthma. N Engl J Med. 2010;363(13):1211-21.
12. Liew FY, Pitman NI, McInnes IB. Disease-associated functions of IL-33: The new kid in the IL-1 family. Nat Rev Immunol. 2010;10(2):103-10.
13. Paragh G, Ugocsai P, Vogt T, Schling P, Kel AE, Tarabin V, et al. Whole genome transcriptional profiling identifies novel differentiation regulated genes in keratinocytes. Exp Dermatol. 2010;19(3):297-301.
14. Radoja N, Gazel A, Banno T, Yano S, Blumenberg M. Transcriptional profiling of epidermal differentiation. Physiol Genomics. 2006;27(1):65-78.
15. Marks GB. House dust mite exposure as a risk factor for asthma: Benefits of avoidance. Allergy. 1998;53(48 Suppl):108-14.
16. Burke H, Leonardi-Bee J, Hashim A, Pine-Abata H, Chen Y, Cook DG, et al. Prenatal and passive smoke exposure and incidence of asthma and wheeze: Systematic review and meta-analysis. Pediatrics. 2012; 129(4):735-44.
17. Zhang J, He X. Significant impact of protein dispensability on the instantaneous rate of protein evolution. Mol Biol Evol. 2005;22(4):1147-55.
18. Heijink IH, Brandenburg SM, Postma DS, van Oosterhout AJ. Cigarette smoke impairs airway epithelial barrier function and cell-cell contact recovery. Eur Respir J. 2012;39(2):419-28.
19. Sano K, Tanihara H, Heimark RL, Obata S, Davidson M, St John T, et al. Protocadherins: A large family of cadherin-related molecules in central nervous system. EMBO J. 1993;12(6):2249-56.
20. Blevins CJ, Emond MR, Biswas S, Jontes JD. Differential expression, alternative splicing, and adhesive properties of the zebrafish delta1-protocadherins. Neuroscience. 2011;199:523-34.
21. Rush J, Moritz A, Lee KA, Guo A, Goss VL, Spek EJ, et al. Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. Nat Biotechnol. 2005;23(1):94-101.
22. Dephoure N, Zhou C, Villen J, Beausoleil SA, Bakalarski CE, Elledge SJ, et al. A quantitative atlas of mitotic phosphorylation. Proc Natl Acad Sci U S A. 2008;105(31):10762-7.
23. Rual JF, Venkatesan K, Hao T, Hirozane-Kishikawa T, Dricot A, Li N, et al. Towards a proteome-scale map of the human protein-protein interaction network. Nature. 2005;437(7062):1173-8.
24. Colland F, Jacq X, Trouplin V, Mougin C, Groizeleau C, Hamburger A, et al. Functional proteomics mapping of a human signaling pathway. Genome Res. 2004;14(7):1324-32.
25. Hackett TL, Warner SM, Stefanowicz D, Shaheen F, Pechkovsky DV, Murray LA, et al. Induction of epithelial-mesenchymal transition in primary airway epithelial cells from patients with asthma by transforming growth factor-beta1. Am J Respir Crit Care Med. 2009;180(2):122-33.

26. Hackett TL. Epithelial-mesenchymal transition in the pathophysiology of airway remodelling in asthma. Curr Opin Allergy Clin Immunol. 2012;12(1):53-9.
27. Johnson JR, Roos A, Berg T, Nord M, Fuxe J. Chronic respiratory aeroallergen exposure in mice induces epithelial-mesenchymal transition in the large airways. PLoS One. 2011;6(1):e16175.
28. Ashcroft GS, Yang X, Glick AB, Weinstein M, Letterio JL, Mizel DE, et al. Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. Nat Cell Biol. 1999;1(5):260-6.
29. Flanders KC, Major CD, Arabshahi A, Aburime EE, Okada MH, Fujii M, et al. Interference with transforming growth factor-beta/ Smad3 signaling results in accelerated healing of wounds in previously irradiated skin. Am J Pathol. 2003;163(6):2247-57.
30. Redies C, Vanhalst K, Roy F. Delta-protocadherins: Unique structures and functions. Cell Mol Life Sci. 2005;62(23):2840-52.
31. Vanhalst K, Kools P, Staes K, van Roy F, Redies C. Delta-protocadherins: A gene family expressed differentially in the mouse brain. Cell Mol Life Sci. 2005;62(11):1247-59.
32. Yoshida K, Yoshitomo-Nakagawa K, Seki N, Sasaki M, Sugano S. Cloning, expression analysis, and chromosomal localization of BH-protocadherin (PCDH7), a novel member of the cadherin superfamily. Genomics. 1998;49(3):458-61.
33. Strehl S, Glatt K, Liu QM, Glatt H, Lalande M. Characterization of two novel protocadherins (PCDH8 and PCDH9) localized on human chromosome 13 and mouse chromosome 14. Genomics. 1998;53(1):81-9.
34. Li X, Howard T, Moore W, Ampleford E, Li H, Busse W, et al. Meta-analyses of genome-wide association studies in five asthma populations identify novel genes associated with lung function. ATS meeting abstract, A6176. 2011.
35. Ross AJ, Dailey LA, Brighton LE, Devlin RB. Transcriptional profiling of mucociliary differentiation in human airway epithelial cells. Am J Respir Cell Mol Biol. 2007;37(2):169-85.
36. Koning H, Sayers I, Stewart CE, de Jong D, Ten Hacken NH, Postma DS, et al. Characterization of protocadherin-1 expression in primary bronchial epithelial cells: Association with epithelial cell differentiation. FASEB J. 2012;26(1):439-48.
37. Koppelman GH, Meyers DA, Howard TD, Zheng SL, Hawkins GA, Ampleford EJ, et al. Identification of PCDH1 as a novel susceptibility gene for bronchial hyperresponsiveness. Am J Respir Crit Care Med. 2009; 180(10):929-35.
38. Sood A, Petersen H, Blanchette CM, Meek P, Picchi MA, Belinsky SA, et al. Methylated genes in sputum among older smokers with asthma. Chest. 2012 Feb 16.
39. Altschuler Y, Hodson C, Milgram SL. The apical compartment: Trafficking pathways, regulators and scaffolding proteins. Curr Opin Cell Biol. 2003;15(4):423-9.
40. Ryniers F, Stove C, Goethals M, Brackenier L, Noe V, Bracke M, et al. Plasmin produces an E-cadherin fragment that stimulates cancer cell invasion. Biol Chem. 2002;383(1):159-65.
41. Haas IG, Frank M, Veron N, Kemler R. Presenilin-dependent processing and nuclear function of gamma-protocadherins. J Biol Chem. 2005;280(10):9313-9.
42. Marambaud P, Shioi J, Serban G, Georgakopoulos A, Sarner S, Nagy V, et al. A presenilin-1/gamma-secretase cleavage releases the E-cadherin intracellular domain and regulates disassembly of adherens junctions. EMBO J. 2002;21(8):1948-56.
43. Kim W, Bennett EJ, Huttlin EL, Guo A, Li J, Possemato A, et al. Systematic and quantitative assessment of the ubiquitin-modified proteome. Mol Cell. 2011;44(2):325-40.
44. Demontis F, Habermann B, Dahmann C. PDZ-domain-binding sites are common among cadherins. Dev Genes Evol. 2006;216(11):737-41.
45. Urquhart P, Pang S, Hooper NM. N-glycans as apical targeting signals in polarized epithelial cells. Biochem Soc Symp. 2005;(72)(72):39-45.
46. Lobas MA, Helsper L, Vernon CG, Schreiner D, Zhang Y, Holtzman MJ, et al. Molecular heterogeneity in the choroid plexus epithelium: The 22-member gamma-protocadherin family is differentially expressed, apically localized, and implicated in CSF regulation. J Neurochem. 2012;120(6):913-27.
47. Krahn MP, Rizk S, Alfalah M, Behrendt M, Naim HY. Protocadherin of the liver, kidney, and colon associates with detergent-resistant membranes during cellular differentiation. J Biol Chem. 2010;285(17):13193-200.
48. Goldberg M, Wei M, Tycko B, Falikovich I, Warburton D. Identification and expression analysis of the human mu-protocadherin gene in fetal and adult kidneys. Am J Physiol Renal Physiol. 2002;283(3):F454-63.
49. Unterseher F, Hefele JA, Giehl K, De Robertis EM, Wedlich D, Schambony A. Paraxial protocadherin coordinates cell polarity during convergent extension via rho A and JNK. EMBO J. 2004;23(16):3259-69.
50. Saburi S, Hester I, Goodrich L, McNeill H. Functional interactions between fat family cadherins in tissue morphogenesis and planar polarity. Development. 2012;139(10):1806-20.

51. Descargues P, Sil AK, Sano Y, Korchynskyi O, Han G, Owens P, et al. IKKalpha is a critical coregulator of a Smad4-independent TGFbeta-Smad2/3 signaling pathway that controls keratinocyte differentiation. Proc Natl Acad Sci U S A. 2008;105(7):2487-92.
52. Davies DE. The role of the epithelium in airway remodeling in asthma. Proc Am Thorac Soc. 2009;6(8):678-82.
53. Kariyawasam HH, Semitekolou M, Robinson DS, Xanthou G. Activin-A: A novel critical regulator of allergic asthma. Clin Exp Allergy. 2011;41(11):1505-14.
54. Lambrecht BN, Hammad H. The airway epithelium in asthma. Nat Med. 2012;18(5):684-92.
55. Xiao C, Puddicombe SM, Field S, Haywood J, Broughton-Head V, Puxeddu I, et al. Defective epithelial barrier function in asthma. J Allergy Clin Immunol. 2011 Jul 11.
56. Dekkers BG, Maarsingh H, Meurs H, Gosens R. Airway structural components drive airway smooth muscle remodeling in asthma. Proc Am Thorac Soc. 2009;6(8):683-92.
57. Munthe-Kaas MC, Willemse BWM, Koppelman GH. Chapter 9: Genetics and epigenetics of childhood asthma. In: European Respiratory Society Monographs, Peadiatric Asthma, ; 2012. Vol. 56, p97-114.
58. Koppelman GH, Nawijn MC. Recent advances in the epigenetics and genomics of asthma. Curr Opin Allergy Clin Immunol. 2011;11(5):414-9.
59. Torgerson DG, Ampleford EJ, Chiu GY, Gauderman WJ, Gignoux CR, Graves PE, et al. Meta-analysis of genome-wide association studies of asthma in ethnically diverse north american populations. Nat Genet. 2011; 43(9):887-92.
60. Hirota T, Takahashi A, Kubo M, Tsunoda T, Tomita K, Doi S, et al. Genome-wide association study identifies three new susceptibility loci for adult asthma in the japanese population. Nat Genet. 2011;43(9):893-6.
61. Moffatt MF, Kabesch M, Liang L, Dixon AL, Strachan D, Heath S, et al. Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. Nature. 2007;448(7152):470-3.
62. Franke L, Jansen RC. eQTL analysis in humans. Methods Mol Biol. 2009;573:311-28.

Chapter 8

Nederlandse Samenvatting

Nederlandse Samenvatting

Astma is een chronische ziekte van de luchtwegen, die gekenmerkt wordt door een ontstekingsreactie en blijvende veranderingen in de structuur van de luchtwegen. Astma patiënten ondervinden periodes van hoesten, piepende ademhaling, en kortademigheid. Daarnaast hebben astmapatiënten een overgevoeligheid van de luchtwegen (bronchiale hyperreacitiviteit) voor factoren zoals rook of mist, of voor allergenen zoals huisstofmijt. Verder kan een verminderde doorgankelijkheid van de luchtwegen door samentrekken van de spieren rondom de luchtwegen, zwelling van het slijmvlies en/of de productie van veel slijm (mucus) leiden tot hevige aanvallen van benauwdheid. Astma wordt vaak gekenmerkt door een allergische reactie tegen specifieke geïnhaleerde stoffen, de zogenaamde allergenen. Bij het eerste contact met deze allergenen worden cellen van het afweersysteem geactiveerd, die onder andere afweerstoffen aanmaken die deze allergenen specifiek herkennen, en eraan kunnen binden. Deze antistoffen zijn de zogenaamde IgE-antilichamen en nestelen zich op mestcellen in de longen. Bij hernieuwd contact worden deze allergenen gebonden aan de IgE-antilichamen op de mestcellen. Hierdoor worden de mestcellen geactiveerd, wat leidt tot de uitscheiding van vele stoffen zoals histamine. Histamine zorgt ervoor dat spiercellen rondom de luchtwegen samentrekken, wat kan leiden tot luchtwegvernauwing en de eerder genoemde symptomen van benauwdheid. Continue blootstelling aan de allergenen kan een chronische ontstekingsreactie van de luchtwegen veroorzaken, wat kan leiden tot blijvende veranderingen van de structuur van de luchtwegen.

Astma is een complexe ziekte die wordt veroorzaakt door een combinatie van genetische factoren en omgevingsfactoren zoals allergenen en sigarettenrook. De genetische factoren die een rol spelen bij astma zijn maar ten dele bekend. De genetische informatie opgeslagen in het DNA van iedere lichaamscel, dat bestaat uit een combinatie van de letters of basen A C T en G, verschilt van persoon tot persoon. Uit voorgaande studies is bekend dat de gevoeligheid om astma te ontwikkelen deels genetisch is bepaald: door verschillen in het DNA hebben sommige mensen meer kans om astma te ontwikkelen dan andere mensen. Om te achterhalen welke verschillen in het DNA nu bijdragen aan een grotere kans op astma worden er vele genetische onderzoeken gedaan, zoals associatie studies. Deze studies bekijken of bepaalde stukken DNA overerven binnen families, oftewel geassocieerd zijn met de ziekte. Ze wijzen dus bepaalde regionen aan waar genen liggen die mogelijk astma of één van de kenmerken van astma zoals bronchiale hyperreactiviteit kunnen veroorzaken.

Vervolgens wordt onderzocht of DNA variaties in bepaalde genen vaker voorkomen bij mensen met astma dan bij gezonde mensen. In de Nederlandse populatie is op deze manier *Protocadherine-1* ontdekt als nieuw gen voor bronchiale hyperreactiviteit en astma. De functie van dit gen en het corresponderende eiwit is grotendeels onbekend. Het doel van dit proefschrift is dan ook om de rol van Protocadherine-1 (PCDH1) bij de ontwikkeling van astma te onderzoeken.

In hoofdstuk 2 beschrijven we de ontdekking van *Protocadherine-1* als een nieuw gevoeligheidsgen voor bronchiale hyperreactiviteit en astma. Eerst werden associatie studies uitgevoerd om te ontdekken welke DNA regio's overerven met astma en bronchiale hyperreactiviteit in de Nederlandse populatie. Er werd een regio op het vijfde chromosoom gevonden die specifiek overerfde met astma en bronchiale hyperreactiviteit. Er was zelfs een sterkere associatie met astma van dit stukje DNA in families die waren blootgesteld aan sigarettenrook. Omdat dit een groot stuk DNA betreft waar meerdere genen liggen is het in meer detail verder onderzocht. Dit werd gedaan door een systematisch onderzoek van bekende basepaar veranderingen van het DNA (bijvoorbeeld een A naar een C of een G naar een T) in dit gebied. Er bleek dat bepaalde basepaar veranderingen gelegen in het *Protocadherine-1* gen vaker voor kwamen bij astma patiënten dan bij gezonde mensen. Deze associatie van *PCDH1* met astma werd bevestigd door genetische studies in populaties uit Engeland en de Verenigde Staten. Met deze studies werd aangetoond dat het *Protocadherine-1* gen is geassocieerd met astma. Dit betekent dat een bepaalde variant van dit gen meer voorkomt bij mensen met astma, terwijl een andere variant van dit gen meer voorkomt bij mensen die geen astma hebben.

Protocadherine-1 behoort tot de familie van adhesiemoleculen. Deze moleculen zorgen ervoor dat naast elkaar gelegen cellen met elkaar verbonden blijven. Protocadherine-1 heeft een relatief zwakke adhesie functie, dus het blijft onduidelijk of adhesie de enige functie van PCDH1 is. We hebben vervolgens onderzocht in welke celtypes van de long PCDH1 eiwit moleculen aanwezig zijn. PCDH1 eiwit moleculen komen vooral voor in cellen van de luchtwegbekleding, zogenaamde epitheelcellen, en specifiek aan de bovenkant (apicaal) van deze cellen (Figuur 1A). Epitheelcellen vormen de eerste barrière tegen ingeademde stoffen, zoals allergenen (bv graspollen, huisstofmijt), luchtvervuilingen en ziekteverwekkers. Adhesiemoleculen zorgen ervoor dat deze cellen sterk tegen elkaar aanliggen. Onze hypothese is dat PCDH1 bijdraagt aan het in stand houden van deze barrière, en dat een fout in het PCDH1 gen of eiwit een zwakkere barrièrefunctie tot gevolg heeft, zodat allergenen en

ziekteverwekkers gemakkelijker kunnen binnendringen. Dit zou uiteindelijk kunnen leiden tot een verhoogde gevoeligheid voor het ontwikkelen van bronchiale hyperreactiviteit en astma, al speelt daar ook de omgeving nog een belangrijke rol in (zoals door blootstelling aan de allergenen en ziekteverwekkers).

Zoals eerder genoemd hebben we bepaalde gebieden op het DNA gevonden waar genen liggen die astma en bronchiale hyperreactiviteit kunnen veroorzaken. Deze studies zijn ook uitgevoerd voor de huidziekte eczeem. Een regio op chromosoom 5 (5q31-33), waarin ook *PCDH1* ligt, is zowel geassocieerd met astma en bronchiale hyperreactiviteit, als met eczeem. In hoofdstuk 3 hebben wij daarom onderzocht of basepaar veranderingen van *PCDH1* die eerder geassocieerd waren met astma en BHR ook associëren met eczeem. Dit hebben we onderzocht in twee verschillende populaties waar vanaf de geboorte het ontstaan van eczeem gevolgd is, en ook DNA afgenomen is (KOALA en PIAMA geboorte cohorten). Een drie basenparen deletie (IVS3-116) van het *PCDH1*-gen bleek zowel in PIAMA als in KOALA geassocieerd te zijn met eczeem. Eczeem is een ziekte van de huid. Als de huid wordt vergeleken met een stenen muur, waarin de stenen de verschillende cellen van de huid voorstellen, dan is bij eczeem het cement tussen de stenen verzwakt of verdwenen. Hierdoor is de barrièrefunctie verminderd en is er een grotere doorlaatbaarheid voor mogelijk gevaarlijke stoffen van buitenaf. Dit manifesteert zich uiteindelijk in een allergische jeukende huidreactie. PCDH1 komt ook voor in huid-epitheelcellen. Wij denken dat PCDH1 zoals bij astma, ook bij eczeem kan bijdragen aan de integriteit van de huidbarrière, en zo aan de gevoeligheid voor het ontwikkelen van de aandoening.

Uit onderzoek van hoofdstuk 2 is gebleken dat PCDH1 tot expressie komt in cellen van de luchtwegbekleding. Het gen *protocadherine-1* is gelokaliseerd op het vijfde chromosoom. Een gen is een stuk DNA dat bestaat uit exonen en intronen. De exonen bevatten de code die nodig is om eiwit moleculen te maken en wordt ook wel coderend DNA genoemd. De intronen bevatten vaak regulerende DNA codes en zijn niet coderend voor een eiwit. Om eiwitmoleculen van een bepaald gen te maken zijn eerst sjablonen nodig die de DNA code naar eiwit productiefabrieken brengen. Deze sjablonen zijn zogenoemde messenger RNAs (mRNAs) en zijn exacte kopieën van de coderende DNA sequenties, behalve dat de letter T door een U vervangen is. Dit afschrijven van de DNA sequentie naar mRNA sequentie wordt ook wel transcriptie genoemd (zie Figuur 1A). De mRNAs komen uiteindelijk aan in de eiwit productiefabrieken. De bouwstenen van eiwitten zijn aminozuren,

waarvan er 20 verschillende bestaan. Aminozuren kan je vergelijken met de kralen van een ketting. Een combinatie van drie mRNA basen (bijvoorbeeld AUG) staat voor één aminozuur of kraal. Zo wordt het hele mRNA molecuul in de fabriek vertaald naar een eiwit-molecuul en worden de bijbehorende aminozuren aan elkaar gekoppeld, wat resulteert in een ketting van aminozuren. Dit proces wordt ook wel translatie genoemd (zie Figuur 1B). De ketting van aminozuren kan gezien worden als een lange streng. Deze ketting wordt uiteindelijk nog in elkaar gevouwen om tot het uiteindelijke eiwit molecuul te komen, wat resulteert in een eiwit-molecuul met verschillende delen die ieder een eigen functie hebben, de functionele domeinen. De expressie van een gen refereert dus naar de aanwezigheid van mRNA of eiwit moleculen waarvoor dat gen codeert in een bepaalde cel.

Van *PCDH1* waren twee mRNA varianten bekend; een variant met 3 exonen (variant 1) en een variant met 5 exonen (variant 2). Beide varianten coderen voor een eiwit met zeven extracellulaire domeinen, terwijl de extra 2 exonen van variant 2 coderen voor geconserveerde intracellulaire domeinen die niet aanwezig zijn in de drie exon variant van het *PCDH1* mRNA. De extracellulaire domeinen lijken sterk op de domeinen van adhesiemoleculen, terwijl de functie van intracellulaire domeinen tot nog toe onbekend is. In hoofdstuk 4 hebben we onderzocht of er nieuwe of veranderde mRNA varianten van *PCDH1* tot expressie komen in epitheelcellen van astma patiënten. Verder hebben we onderzocht wat er met de expressie niveaus van PCDH1 gebeurd tijdens de uitrijping van luchtwegepitheelcellen (differentiatie) die wordt bereikt door de epitheelcellen tijdens weefselkweek in het laboratorium bloot te stellen aan lucht, net zoals dat in het lichaam gebeurt. Hierbij hebben we een aantal interessante observaties gedaan. Ten eerste vonden we nieuwe exonen aan de 5' kant van het *PCDH1*-gen ('links' van exon 1: voor het begin van het gen) die verschillend gebruikt worden door isovorm 1 en 2. Dit kan er op duiden dat de twee varianten verschillend gereguleerd worden. Ten tweede vonden we varianten die geconserveerde intracellulaire domeinen missen. Ten derde vonden we een nieuwe variant dat exon 1 en een deel van exon 2 mist en daardoor slechts twee extracellulaire domeinen en de intracellulaire domeinen tot expressie brengt. Deze isovorm hebben we isovorm 3 genoemd. De hierboven beschreven resultaten geven de complexiteit van het *PCDH1* gen weer, en tonen verschillende niveaus aan waarop *PCDH1* gereguleerd kan worden (Figuur 1B). Een belangrijke bevinding van hoofdstuk 4 was dat zowel de mRNA als eiwit expressieniveaus van PCDH1 toenamen tijdens het uitrijpen (differentiatie) van luchtweg epitheelcellen. Dit zou kunnen betekenen dat PCDH1 een belangrijke rol speelt tijdens dit proces, maar verder onderzoek is nodig om de exacte rol van PCDH1 tijdens differentiatie te bepalen.

Astma is een ziekte dat zowel door genetische als omgevingsfactoren veroorzaakt wordt. Bekend is wel, dat blootstelling aan omgevingsfactoren zoals sigarettenrook en huisstofmijt een versterkte hyperreactiviteit bij astmapatiënten geven. *PCDH1* blijkt in ons onderzoek bovendien sterker geassocieerd met bronchiale hyperreactiviteit en astma in families die zijn blootgesteld aan sigarettenrook, dan in families die niet zijn blootgesteld aan sigarettenrook. Daarom hebben we in hoofdstuk 5 onderzocht of PCDH1 gereguleerd wordt door sigarettenrook en huisstofmijt in verschillende muismodellen. Eerst hebben we de muis en humane protocadherine-1 genen vergeleken. We vonden een erg hoge overeenkomst tussen mens en muis PCDH1, zowel op mRNA als op eiwit niveau. Bovendien vonden we in de muizenlong ook een derde isovorm, die alleen het intracellulaire stuk bevat dat gedeeld wordt met isovorm 2. Onderzoek van muis *Pcdh1* kan dus een goed model zijn van het menselijke *PCDH1*.

Vervolgens hebben we de invloed van huisstofmijt blootstelling op Protocadherine-1 expressie onderzocht. Op ons laboratorium is een huisstofmijt muismodel voor astma gevalideerd. Onder invloed van directe huisstofmijt toediening in de luchtwegen van de muis veranderen de epitheelcellen in de muizenlong naar slijmbeker cellen die mucus produceren. Bovendien vindt er een ontstekingsreactie plaats en ontwikkelen de muizen luchtweg hyperreactiviteit. Wij vonden een verlaagde protocadherine-1 eiwitexpressie in de luchtwegen van de muis die blootgesteld waren aan huisstofmijt. Na een rustperiode van twee weken waarin geen huisstofmijt was toegediend zagen wij weer een toename van *Pcdh1* mRNA expressie niveaus. Dus: blootstelling aan huisstofmijt kan Protocadherine-1 expressie in de luchtwegen verlagen, dat later weer toeneemt in de herstelfase van het epitheel. Muizen die zijn blootgesteld aan sigarettenrook krijgen een ontstekingsreactie in de longen en ontwikkelen ook luchtweg overgevoeligheid. Wij zagen een directe afname van *Pcdh1* mRNA expressie in de longen van muizen die waren blootgesteld aan sigarettenrook. Een langere blootstelling zorgde voor een verdere verlaging van deze expressieniveaus. Sigarettenrook kan de epitheliale barrière verzwakken door het verbreken van de contacten tussen cellen. Wij stellen voor dat mensen die zijn blootgesteld aan sigarettenrook en die drager zijn van base-veranderingen in het *PCDH1*-gen, uiteindelijk bronchiale hyperreactiviteit en astma ontwikkelen door een verlaging van de expressieniveaus van PCDH1 en epitheliale adhesiemoleculen dat leidt tot een verlaagde barrièrefunctie.

Protocadherine-1 behoort tot de familie van adhesiemoleculen. Omdat Protocadherine-1 een relatief zwakke adhesiefunctie heeft, is het de vraag of PCDH1 ook nog andere functies uitoefent. In twee studies is beschreven dat het PCDH1 eiwitmolecuul mogelijk een fysieke

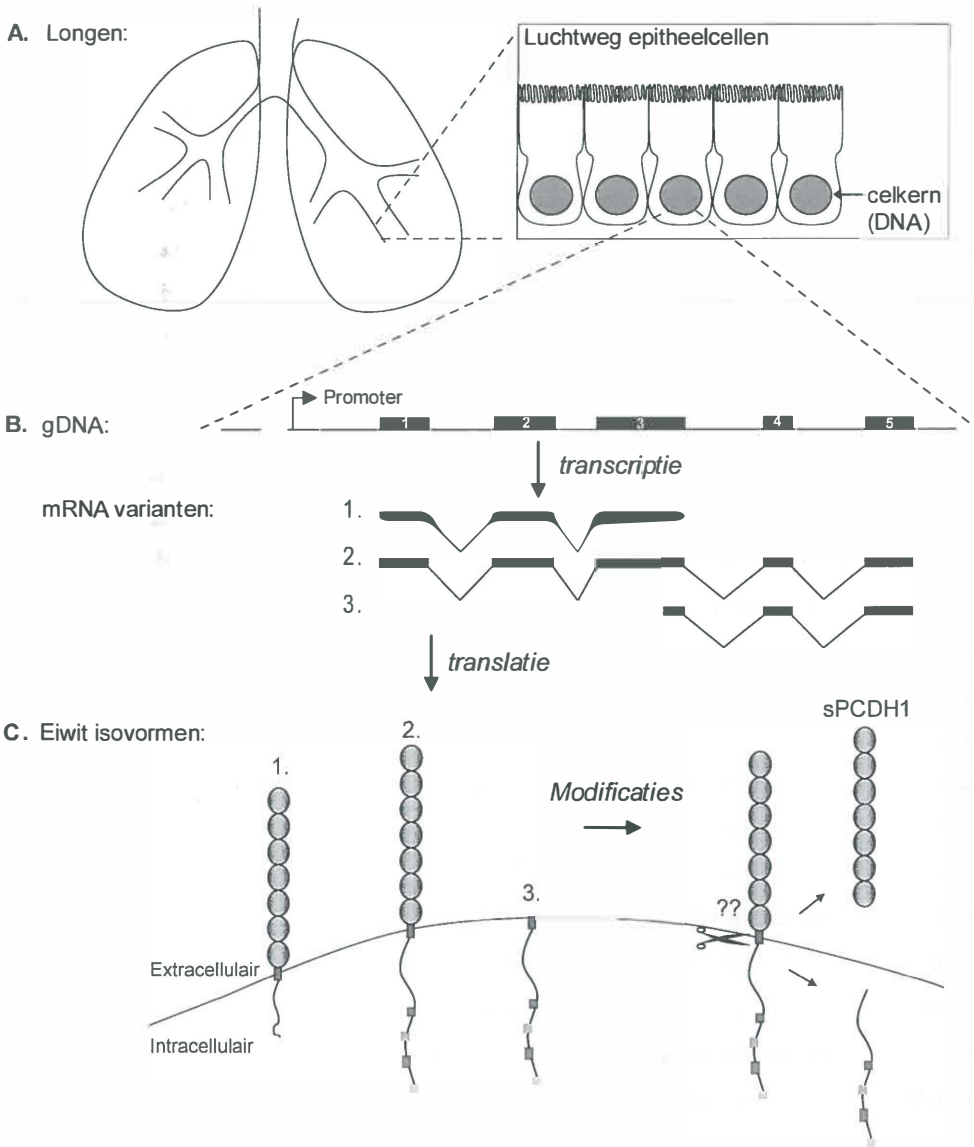
interactie aangaat met de signaalmolecuul SMAD3. SMAD3 is een molecuul dat signalen doorgeeft van membraangebonden Transforming Growth Factor β (TGFβ) – receptoren, die geactiveerd zijn door de groeifactor TGFβ, naar de kern van de cel. In de kern activeert SMAD3 de transcriptie van verschillende genen. Daardoor kunnen processen plaatsvinden zoals: (1) herstel van epitheel na schade en, (2) de verandering (transitie) van een epitheelcel naar een bindweefsel of spiercel (mesenchymale cel) die uit de epitheellaag kan migreren (EMT). Verder is gebleken dat SMAD3 een rol kan spelen in differentiatie van epitheelcellen. In hoofdstuk 6 vragen we ons af of PCDH1 ook als signaal molecuul zou kunnen functioneren. Daarom hebben we de lokalisatie van PCDH1 isovormen in verschillende compartimenten van de bronchiale cellijn 16HBE onderzocht en hun fysieke interactie met de signaalmolecuul SMAD3. We vonden een nieuw eiwitproduct van PCDH1 dat wordt uitgescheiden door de epitheelcellen (sPCDH1). Dit eiwitproduct heeft geen intracellulaire domeinen en bestaat dus alleen uit extracellulaire domeinen. Verder vonden we een reeks kleinere eiwitproducten die alleen uit intracellulaire domeinen bestaan. Het mechanisme dat verantwoordelijk is voor het ontstaan van deze extracellulaire sPCDH1 en intracellulaire eiwit producten is onbekend. Ten eerste kunnen deze producten zijn ontstaan door het knippen van de complete isovormen 1 en / of 2 door specifieke knipenzymen zoals metalloproteases. Ten tweede is het een mogelijkheid dat deze eiwit fragmenten zijn ontstaan door alternatieve transcriptie, zoals voor isovorm 3. Als laatste bestaat de mogelijkheid dat deze eiwitproducten zijn ontstaan door afbraak van de complete isovormen 1, 2 en / of 3 (Figuur 1C). Omdat een deel van de intracellulaire eiwitfragmenten specifiek naar de kernmembraan lokaliseren, zouden ze bij kunnen dragen aan signalering. Het mechanisme achter het ontstaan van deze fragmenten en de mogelijke signaleringsfuncties behoeft verder onderzoek.

In hoofdstuk 6 hebben we ook onderzocht of PCDH1 een fysieke interactie aangaat met SMAD3 in de 16HBE cellijn. Door PCDH1 met een specifiek antilichaam uit de cellen te vissen vonden we dat SMAD3 meekwam met het 'opgeviste' PCDH1. Via deze procedure vonden we zowel een interactie van beide isovormen van PCDH1 met SMAD3 als andersom. Dit is bewijs voor een directe interactie tussen SMAD3 en PCDH1, en opent nieuwe mogelijkheden voor onderzoek naar de functie van het PCDH1 eiwit. Door een interactie aan te gaan met SMAD3 zou PCDH1 het herstel van een beschadigde epitheellaag, of de transitie van epitheelcellen naar migrerende mesenchymale cellen (EMT) en differentiatie van epitheelcellen kunnen beïnvloeden, allemaal processen die worden gereguleerd door de groeifactor TGFβ. Het herstel van een beschadigde epitheellaag (wond) bestaat uit verschillende fasen. Ten eerste zullen de gezonde epitheelcellen die de wond omringen

moeten de-differentiëren. Hierbij verliezen deze cellen hun functie als epitheelcel en verkrijgen ze een migrerende functie. Dit proces is vergelijkbaar aan EMT. Vervolgens kunnen ze zich gaan verplaatsen naar de plaats van de wond. Uiteindelijk moeten deze cellen weer differentiëren om weer een functionele epitheelcel te vormen. PCDH1 zou een rol kunnen spelen in al deze fases van epitheel herstel. Wij gaan uit van de hypothese dat mensen met astma die drager zijn van basepaar veranderingen van het *PCDH1* gen een verminderd functioneren of verlaagde expressieniveaus van PCDH1 hebben. Daardoor worden processen als adhesie en differentiatie ontregeld, zodat het epitheel slecht herstelt na schade en epitheelcellen niet goed differentiëren. Dit kan leiden tot een epitheellaag die als het ware in een reparatiefase blijft hangen. Vervolgens raakt de epitheelbarrière verzwakt, wat uiteindelijk kan leiden tot bronchiale hyperreactiviteit en astma.

Concluderend, Protocadherine-1 is een gen dat zowel geassocieerd is met astma als met eczeem. PCDH1 komt hoog tot expressie in de bekledende cellen van de luchtwegen: het luchtwegepitheel. De transcriptie van *PCDH1* resulteert in verschillende mRNA varianten, waarbij grote verschillen zijn waargenomen in de expressie van sequenties die coderen voor de intracellulaire domeinen. Verder nemen de expressieniveaus van PCDH1 toe tijdens het uitrijpen (de differentiatie) van epitheelcellen. Blootstelling van muizen aan omgevingsfactoren zoals huisstofmijt en sigarettenrook verlaagt de expressie van PCDH1 in de longen van muizen. PCDH1 eiwit isovormen zijn ontdekt zowel buiten de cel (uitgescheiden) als in de kern van de cel. Verder heeft PCDH1 een fysieke interactie met SMAD3. Deze resultaten kunnen er op duiden dat PCDH1 niet alleen een adhesiemolecuul is, maar ook functioneert als een signaleringsmolecuul. Wij denken dat, door middel van de interactie met SMAD3 en door het induceren van expressieniveaus gedurende differentiatie van het epitheel, PCDH1 een rol kan spelen in het herstel en de differentiatie van het luchtwegepitheel. In mensen die drager zijn van base veranderingen in het *PCDH1* gen, zou een verminderde functie van PCDH1 dus kunnen leiden tot een ontregeld herstel en ontwikkeling van het epitheel. Dit kan leiden tot een zwakkere epitheelbarrière, waardoor allergenen en andere omgevingsfactoren gemakkelijker kunnen binnendringen en vervolgens een chronische allergische reactie veroorzaken, wat uiteindelijk kan leiden tot bronchiale hyperreactiviteit en astma. Ons uiteindelijke doel is om medicatie te ontwikkelen die gericht is op het herstel van de PCDH1 functie waarmee de epitheelbarrière gestabiliseerd kan worden en weer volledige bescherming biedt.

Figuur 1: Overzicht PCDH1 expressie, gen, mRNA en eiwit isovormen
De luchtwegen in onze longen zijn bekleed met epitheelcellen, waarin Protocadherine-1 tot expressie komt. Elke cel heeft een celkern waarin het DNA zeer goed opgevouwen is (A). Het genomisch DNA (gDNA) bestaat uit verschillende genen die aangezet kunnen worden door een promoter. Elk gen bestaat uit exonen (zwarte box) en tussengelegen intronen. Bij transcriptie van het PCDH1 gen worden de vijf PCDH1 exonen afgeschreven in verschillende volgordes (transcriptie). De intronen worden later verwijderd, wat resulteert in drie PCDH1 mRNA varianten (B). Vervolgens worden de verschillende varianten weer vertaald naar eiwit isovormen (translatie). Deze eiwit moleculen kunnen mogelijk nog verder gemodificeerd worden, wat zou kunnen leiden tot uitgescheiden PCDH1 (sPCDH1) en intracellulaire producten (C).



Abbreviations

Act	Activin
ADAM	A Disintegrin And Metalloproteinase
ADRB2	β2-adrenergic receptor
AD	Atopic Dermatitis
AEC	3 amino-9-ethylcarbazole
AHR	Airway hyperresponsiveness (mouse)
ALI	Air Liquid Interface
AMP	Adenosine-mono-phosphate
ANOVA	Analysis of variance
ASM	Airway smooth muscle
AUC	Area Under the Curve
BAL	Bronchial Alveolar Lavage
BHR	Bronchial Hyperresponsiveness (human)
CDHR	Cadherin related
cds	Coding sequence
CM	Conserved motif
cM	Centi-Morgan
CS	Cigarette Smoke
Ct-value	Cycle threshold value
CTF	C-terminal fragment
Cyto	Cytoplasmic proteins
DAMPs	Damage Associated Molecular Patterns
Del	Deletion
DCs	Dendritic Cells
ECs	Extracellular Cadherin repeats
ECP	Eosinophilic cationic protein
ECM	Extracellular matrix components
EDC	Epidermal Differentiation Complex
EDN	Eosinophil-derived neurotoxin
EGFR	Epidermal growth factor receptor
EL	Expression level
EMEM	Eagle minimum essential medium
EMT	Epithelial to Mesenchymal Transition

EMTU	Epithelial Mesenchymal Trophic Unit
EPO	Eosinophil-derived peroxidise
ETS	Environmental tobacco smoke
ET-1	Endothelin-1
eQTL	expression Quantitative Trait Locus
FBAT	Family Based Association Tests
FCS	Fetal Calf Serum
FEV_1	Forced Expiratory Volume in one second
FGF	Fibroblast growth factor
FL	Full length
FLG	Filaggrin
GEE	Generalized Estimating Equations
GWAS	Genome Wide Association Study
HDM	House Dust Mite
(H)LOD	Heterogeneity Logarithm Of Odds score
HMGB1	High mobility group box 1
Hs	Homo sapiens
IFN-γ	Interferon gamma
IGF	Insulin-like growth factor
IL	Interleukin
IP	Immunoprecipitation
ISAAC	The International Study of Asthma and Allergies in Childhood
KOALA	Dutch acronym for: Child, Parent, health, Focus on Lifestyle and Predisposition birth cohort
LD	Linkage Disequilibrium
MBP	Major basic protein
MDCK	Madin-Darby Canine Kidney
MHC	Major Histocompability Class
Mm	Mus musculus
M-PER	Mammalian Protein Extraction Reagent buffer
NEC	Neuroendocrine-cell
NK	Natural Killer cells
Nucl	Nuclear proteins
OR	Odds Ratio

PAMPs	Pathogen-Associated Molecular Patterns
PAS	Periodic Acid Schiff
PBS	Phosphate Buffered Saline
PBECs	Primary Bronchial Epithelial Cells
PCDH1	Protocadherin-1
PCDH-LKC	Protocadherin of the Liver, Kidney and Colon
PCR	Polymerase Chain Reaction
PC_{20}	a 20% fall in the FEV_1
PDGF	Platelet-derived growth factor
PDZ-BS	PDZ-domain Binding Site
PIAMA	Prevention and Incidence of Asthma and Mite Allergy birth cohort
PP1α	Protein phosphatase 1 alpha
qRT-PCR	quantitative Reverse Transcriptase PCR
RACE	Rapid Amplification of cDNA Ends
RBM	Reticular basement membrane
Rs	Ref SNP accession ID
SBE	SMAD3 binding element
SDS	Sodium Dodecyl Sulphate
sECad	soluble E-Cadherin fragments
SMAD3	Mothers against decapentaplegic homolog 3
SNPs	Single nucleotide polymorphisms
SP	Signal Peptide
sPCDH1	soluble PCDH1 protein product
TEER	Transepithelial electrical resistance
TGFβ	Transforming Growth Factor β
TLRs	Toll-like receptors
TM	Transmembrane domain
TNF-α	Tumor Necrosis Factor alpha,
Tregs	Regulatory T-cells
UKWP	UK Working Party criteria
UTR	Untranslated Region
ZO-1	Zona-Occludins 1
16HBE	Human Bronchial Epithelial cell line 16HBE 14o-
95% CI	95% Confidence Interval

Dankwoord

Het proefschrift is eindelijk klaar! Voor mij is dit een afsluiting van een periode met vele hoogte maar ook enkele dieptepunten. Na het overlijden van mijn broer stond mijn leven even stil.....Na weer opgekrabbeld te zijn gebeurde drie jaar later aan het eind van mijn aio-periode het ondenkbare. Mijn vader werd ziek en overleed.... Ik wil iedereen bedanken die mij en mijn familie gesteund heeft tijdens deze moeilijke periodes. Daarnaast waren er gelukkig ook veel mooie periodes. Gedurende mijn promotie heb ik veel leuke mensen ontmoet en prettig mee samengewerkt, zowel in binnen als buitenland. Natuurlijk wil ik iedereen bedanken, maar het zijn er teveel om op te noemen en het kan voorkomen dat ik vergeet iemand te vermelden.

Het begon allemaal in maart 2007, toen ik vanuit Wageningen hier naar Groningen kwam. Na eerst tijdelijk forens te zijn geweest, door te reizen vanaf mijn ouderlijk adres in Leeuwarden, vond ik uiteindelijk een kamer niet ver van het UMCG, dus lekker dicht bij het lab. Als eerste wil ik Debora bedanken voor het inwerken in het Protocadherine-1 labwerk. Je hebt me wegwijs gemaakt op de verschillende kweeklaboratoria. Daardoor heb ik veel opgestoken en met name over de wispelturigheid van onze 16HBE cellijn. Resultaten gebaseerd op al je primaire kweekwerk zijn uiteindelijk mooi gepubliceerd in FASEB Journal.

Mijn eerste werkkamer zat vlak na de brug. Dries, Benoit, Hadi & Soheila, bedankt voor de alle gezelligheid op de kamer. We wisten niet dat we daar weg moesten totdat ze plotseling in onze muren gingen boren en het bleek dat ze onze kamer gingen halveren.

Ondertussen ben ik begin 2008 naar Nottingham University in Engeland gegaan om de techniek RACE te leren. Thanks for the warm welcome, Ian and Ceri. Lee, Janie, Emily, Asif, Imran and Javier, thanks for the nice time and parties in Nottingham, and the holiday at Ibiza.

Terug gekomen in het UMCG verhuisden we naar de kamer die uitkeek op het personeelsrestaurant. Helaas was hier geen directe frisse lucht. Inmiddels zaten we daar met zes mensen, omdat Benoit was vertrokken en Dennie en Sijranke er bij waren gekomen. Dat bleek teveel omdat de warmte gegenereerd door deze hardwerkende aio's en de computers niet kon worden afgevoerd. Uiteindelijk zijn Hadi & Soheila naar een andere kamer verhuisd. Met Dennie en Dries heb ik nog veel lol beleefd op deze kamer. Dennie, de volgende keer als we je vastplakken aan de stoel zetten we je toch echt voor de kamer van de prof neer!

We hadden inmiddels een grote groep aio's op het lab. Roland zat op een andere kamer, maar ik denk dat we een aantal prachtige fietstochten hebben beleefd. Ik was blij dat ik jouw achterwiel kon volgen de zaterdagochtend vroeg op weg naar de Winsumer

Wierdentocht, want ik voelde de vrijdagborrel nog wel! We hebben toch maar mooi veel toertochten gefietst met Hans, Maaike en Renée. De vrijdagavondborrel slaan we zeker niet over. Deze labborrel werd eerst de laatste vrijdag van de maand gehouden in de Stadtlander, maar met Jan hebben we toendertijd besloten om de frequentie op te hogen naar een keer per week. Gelukkig waren er altijd wel slachtoffers te vinden die meegingen, zoals Irene, Harold, en de moleculaire farrnacologen Reinoud en Tonio, en later Eef. Het was gezellig discussiëren en borrelen met iedereen. Zulke borrels zijn een goede afwisseling van het labwerk, zoals ook de woensdag – snackdag. Met Marco, Harold, Uilke, en de aio's gezellig het broodje hamburger met kerrie-saus bij Café Prins naar binnen werken. Vervolgens gingen we noodgedwongen naar de wat minder hygiënische brandgevaarlijke Chinese snackbar.

Inmiddels had Debora ons lab verlaten en is Uilke het PCDH1-team komen versterken. Uilke, ik hoop dat je veel van me hebt opgestoken. Samen hebben we vele muizenlongen tot snot verholpen, en vele long coupes gekleurd en intensiteiten gekwantificeerd. Dat was niet altijd het leukste werk. Hopelijk wordt dit werk binnenkort nog geaccepteerd voor publicatie. Ik denk dat we een goed duo vormden, natuurlijk bedankt hiervoor en voor het zijn van mijn paranimf. Machteld en Wim bedankt voor de hulp met het interpreteren van de long kleuringen. Later kwamen ook Mariska en Brigitte ons PCDH1-team versterken. Mariska, bedankt voor het runnen van de vele westerns. Brigitte, wij hebben wat korter samen gewerkt, maar erg bedankt voor je hulp aan met name mijn laatste experimentele hoofdstuk.

De analisten op het lab wil ik natuurlijk ook bedanken: Jacobien, Simone, Lisette, Yo-Theo, Marnix, Marco, Harold, Janneke (wel Liwadders blijven praten hoor!), en uiteraard ook de diagnostiekers. Peter en Harold natuurlijk ook bedankt voor de plezierige werksfeer op het DNA lab. Dat maakte het opwerken van al die kolonies altijd een stuk gezelliger. Een persoon die nog niet in dit rijtje staat, maar specifiek bedank is Renée. Ik ben heel blij dat ik jou ben tegen gekomen en dat je mijn vriendin wilt zijn. Je was en bent mijn steun en toeverlaat. We hebben dieptepunten meegemaakt, maar gaan zeker nog vele mooie dingen beleven samen!

Natuurlijk wil ik ook mijn vrienden uit Leeuwarden bedanken. Jurjen, Jan en Frans, bedankt voor de gezelligheid tijdens de stapavondjes. Frans bedankt voor je vriendschap en de dinsdagavonden met gezellig eten, voetbal kijken en biertjes drinken. Ik ben blij dat je mijn paranimf wilt zijn! Verder was er een welkome ski-afwisseling tijdens de slappe Nederlandse winters. Als schaatsende Fries wist ik niet of ik het echt leuk zou vinden, maar na de eerste vakantie was ik direct verkocht. John, Eline, en Jan, bedankt voor de leuke vakanties.

In de loop van de tijd kwamen er nieuwe aio's bij op verschillende projecten. Daardoor werd de kamerruimte wat krap, en omdat ik bovendien niet veel labwerk meer ging doen verhuisde ik naar de kamer in het Triade-gebouw, het terrein van Néomi. Néomi, sorry dat je geen harde muziek meer kan draaien, maar natuurlijk bedankt voor de gezellige tijd daar en de lunch met een overheerlijk soepje. Met Nicole heb je er weer een goede kamergenoot bij. Als jullie de strenge winters daar overleven komen er vast nog veel mooie IL1RL1 publicaties.

Natuurlijk wil ik ook de aio's die ik korter heb meegemaakt bedanken, Maaike, veel succes binnenkort in Southampton, en Laura en Daan met de immunotherapie en DAMPs. Verder wil ik natuurlijk de afdeling kinderlongziekten bedanken voor hun steun: Eric, Ewoud, Elianne, Bart, Christine, Hilde, Janette, bedankt. Natuurlijk ook de studenten Tanja en Olaf bedankt voor jullie bijdrage aan Protocadherine-1. Verder iedereen bij de GRIAC en Genetics meetings bedankt voor de input. Olga, ik hoop dat ik niet te vervelend werd met het vragen naar de biologische betekenis van de gevonden associaties. Marjan Kerkhof bedankt voor je hulp met de analyses van PCDH1 en eczeem.

Ook wil ik de leescommissie, bestaande uit Prof. John Holloway, Prof. Pieter Hiemstra en Prof. Frans van Roy bedanken voor hun tijd en aandacht aan de beoordeling van dit werk. Verder wil ik ook de sponsors van dit proefschrift bedanken.

Dan zijn we nu aangekomen bij mijn begeleiders. Martijn, de hoeveelheid commentaar die je geeft op een manuscript is volgens mij omgekeerd evenredig met de hoeveelheid wijn in de fles! Ik verheugde me altijd op de 100+ comments....Zeker is dat de artikelen er stukken beter van werden. Dirkje en Antoon, bedankt voor jullie input tijdens alle besprekingen en jullie commentaar op de artikelen! Dirkje, ik vind het erg jammer dat je niet bij de promotie kan zijn, maar wens je natuurlijk veel plezier op je sabattical. Uiteraard wil ik ook Gerard bedanken. Zonder jou was het PCDH1 project er waarschijnlijk niet geweest. Jouw drive om dit gen functioneel te karakteriseren heeft altijd motiverend gewerkt. Het was altijd plezierig samenwerken. Bedankt ook voor het begrip en de ondersteuning in de moeilijkere periodes van mijn aio-periode. Dit heeft mij erg geholpen.

Als laatste wil ik mijn familie bedanken. Mem, Boukje, Chris, Judith, Renee, we hebben samen deze moeilijke periodes doorgemaakt. De komende tijd zal ook niet altijd gemakkelijk zijn, maar met elkaar slaan we ons er wel doorheen!

Zo komt een bijzondere periode tot zijn einde. Al met al hoop ik dat iedereen de samenwerking met mij ook prettig vond. Het ga jullie allemaal goed!